THE INTERACTION OF GM_1 GANGLIOSIDE WITH CHOLERA TOXIN ON MEMBRANES OF CELLS

MARIANNE ELIZABETH FLETCHER

Ph . D

UNIVERSITY OF EDINBURGH

1990

PREFACE

The work reported in this thesis was carried out between 1st October 1985 and the 30th September 1988 under the supervision of Dr. Simon van Heyningen at the Department of Biochemistry, University of Edinburgh Medical School, or under the supervision of Dr. Peter Garland and Dr. Robert Shields at Colworth Laboratory, Unilever Research, Sharnbrook, Bedford. This work was first presented in 1988 and has since been revised resulting in the current composition. Unless otherwise stated all material presented is the sole work of the author, as is the composition.

Marianne Fletcher (nee Parkinson)

ABSTRACT

Cholera toxin (molecular weight 84kD) binds with high affinity (K_d = 10^{-9}M) to GM_1 ganglioside on the outer surface of most eukaryotic cells before all or part of the molecule is internalised and activation of adenylate cyclase occurs. The GM_1 ganglioside is believed to diffuse laterally on the cell surface. There is also evidence to suggest that cholera toxin requires multivalent binding to GM_1 before it can activate adenylate cyclase. The effect of cholera toxin binding on the lateral diffusion of GM_1 was examined using the Fluorescence Recovery after Photobleaching technique either with fluorescently labelled toxin or with inserted, fluorescently labelled GM_1 ganglioside. Both toxin-receptor complex and receptor alone showed the same percentage mobility (about 60-70%) on the surface of the NIH 3T3 cells (a fibroblast cell line) and both had a lateral diffusion coefficient of about 1×10^{-9} cm^2s^{-1}. This result shows that bound toxin mobility does not differ from inserted ganglioside mobility. An interpretation of the results may be that GM_1 molecules were compartmentalized on the fibroblast cell surface into mobile and immobile areas. The involvement of non-coated invaginations in cholera toxin internalisation was confirmed by preliminary binding experiments with colloidal gold conjugated cholera toxin. The cholera toxin was also used as a probe to locate GM_1 intracellularly by the Post-Embedding Immunogold technique on mouse small intestine (target tissue for cholera toxin). A previously unreported, specific binding to the heterochromatin of the nucleus of mouse intestinal cell was discovered. The intracellular localisation of GM_1 has previously mainly been studied by cell fractionation studies which indicated that only a small amount of total cell ganglioside is found within the nucleus. This binding of cholera toxin to the nucleus was further investigated using biochemical binding studies which also appeared to indicate a specific binding site for the toxin within the nucleus which has not been fully characterised.

To My Parents

you have to have the brashness to create,
you have to have the toughness to experiment;
you have to run the risk of failure;
you have to be honest enough to learn from
what happens; you have to be brave enough to
start over; you have to be willing to be
judged on what you contribute .....

..... this is research

(Hainer, R.)

ACKNOWLEDGEMENTS

I would first like to thank my two supervisors, Dr. Simon van Heyningen and Dr. Peter Garland for helpful discussion over the last five years. There are also many other people to thank from both Edinburgh University and Unilever Colworth Laboratory,and other extraneous bodies!

At Edinburgh, my thanks go especially to David Longbottom who has been a good friend and colleague. Also to Tamara, Viv, Margaret, Mohinder and Carol who made laboratory life more sociable.

At Unilever, my thanks go to Dr. Robert Shields who took over the role of provider when Peter Garland left to go to Amersham. I am grateful to Dr. Martin Green for many helpful discussions. I am indebted to Colin Smith for cutting all my e.m. sections and John Birmingham who explained the intricacies of the laser and computed all my FRAP results. The following people at Unilever cannot be left out: Christine Foulger and Anita Evans who grew the NIH 3T3 cells and Alison Hoole who provided me with some delicious home-cooked meals.

Dr. Paul Agutter of Napier College, Edinburgh, must also be thanked for his advice on the nuclear membrane work. Prof. Donald Kelly and Dr. Roger Batt of Liverpool University were very kind to help me with some cholera toxin internalisation experiments.

This work could not have been done without the grant from the SERC and financial support from Unilever. This money also enabled me to attend the 3rd European Bacterial Protein Toxin meeting in Uberlingen in June 1987.

Finally, thank you to my parents and friends for all their moral support and most of all to my husband Mike for the stability he provided during my three years of research and the last two years of revision.

CONTENTS

LIST OF FIGURES Page

LIST OF TABLES PAGE

ABBREVIATIONS

A_1	The active peptide of the A subunit of cholera toxin
ADP-ribose	Adenosine disphospho ribose
BSA	Bovine serum albumin
cAMP	Adenosine 3', 5' - monophosphate (cyclic AMP)
Da	Daltons
DMEM	Dulbeccos modified eagles medium
DMPC	Dimyristoylphoshatidylcholine
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
EDTA	Ethylene Diaminetetraacetic acid
EM	Electron Microscopy
FITC	Fluorescein isothiocyanate isomer I
FRAP	Fluorescence Recovery After Photobleaching
GM_1	Galactosyl-N-acetyl galactosaminyl [N-acteylneuraminyl]-galatosylglycosyl ceramide
HPTLC	High performance thin layer chromatography
IMP	Implementation program
NAD +	Nicotinamide adenine dinucleotide
NBF-Chloride	4-chloro-7-nitrobenzofuran
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (Dulbeccos)
PMT	Photomultiplier tube
SAPU	Scottish antibody production unit
SDS	Sodium dodecyl sulphate
TLC	Thin layer chromatography
TWEEN	Polyethylene (20) - sorbitan monolaurate
UV	Ultra violet
V/V	Volume/volume
W/V	Weight/volume

Chapter 1

INTRODUCTION

INTRODUCTION

1.1.0 BACKGROUND: CHOLERA TOXIN

It was not until 1969 that cholera enterotoxin was isolated by Finkelstein et al., over a century after the comma-shaped bacterium Vibrio cholerae had been identified by Paccinni (see Pollitzer, 1965). The symptoms of the disease are a massive diarrhoea, often followed by vomiting, which can deplete the victim of up to twenty-five per cent of body fluids and essential salts within hours. The most common cause of death is an intense peripheral vasoconstriction (De, 1961).

The V. cholerae are spread by human excrement in contaminated drinking water. The bacteria thrive in the digestive tract, where they produce the protein enterotoxin, which is now known to be a major cause of the disease symptoms. Thanks to the great improvement in sanitation, the disease has been eradicated in developed countries, but it is still a major killer in the Third World (Garfield, 1986).

Although the toxin molecule has been very widely studied, the details of its mechanism of action on the gut remain obscure.

1.1.1 Nature of the Toxin

Cholera toxin is a globular protein, with a molecular weight of about 84 000 Daltons, containing no appreciable amount of lipid or sugar. (Sattler et al., 1975; LoSpalluto and Finkelstein, 1972; van Heyningen, 1976). The toxin molecule has an isoelectric point of 6.9 and has a very stable structure, maintaining its activity even after boiling in sodium dodecyl sulphate (Lai, 1980).

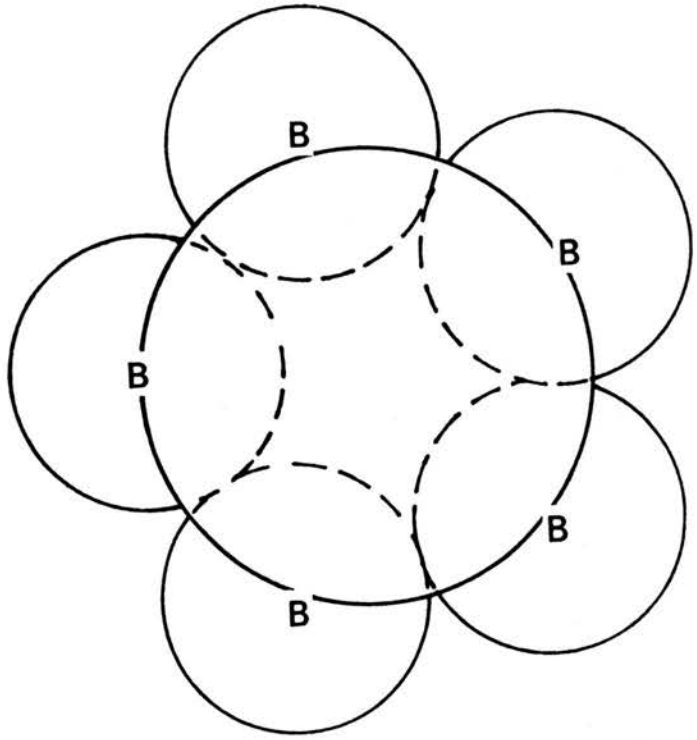
Often secreted with cholera toxin is a non toxic protein termed choleragenoid, which has a molecular weight of about 55 000 Daltons and is an incomplete cholera toxin molecule. The function of choleragenoid, if any, is unknown.

1.1.1.1 Subunit Structure of the Toxin

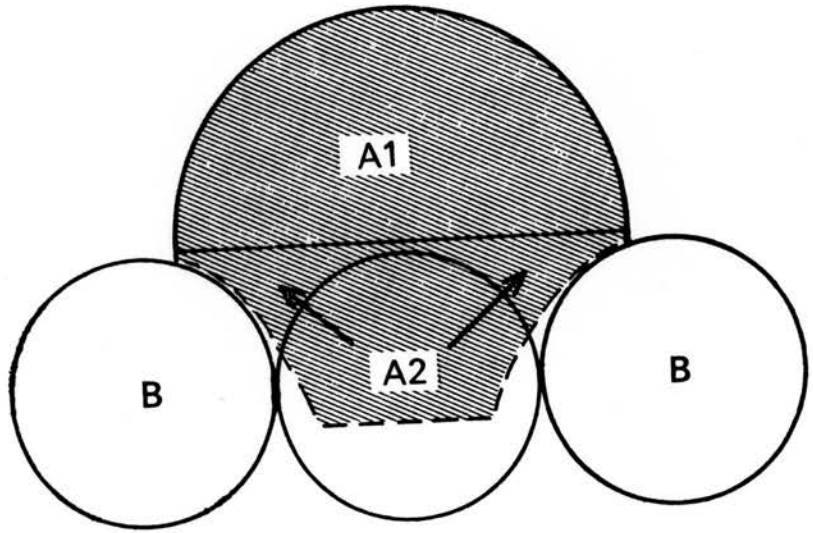
Initial analysis of the toxin molecule by SDS polyacrylamide gel electrophoresis showed that it is composed of two non-covalently linked subunits termed A and B (Finkelstein et al., 1972). Molecular weight determinations of each of the subunits and the whole toxin suggested a molar ratio of five B subunits to every A subunit (Lai et al., 1977). This ratio has been verified by cross-linking experiments (van Heyningen, 1977; Gill, 1977).

Fig.1.1

Diagrammatic representation of a cholera toxin molecule (drawn roughly to scale). Reproduced with permission from van Heyningen, 1977.



Plan



Elevation

Electron microscopy also supports the evidence for the molar ratio of B subunits (Sigler et al., 1977). X-ray crystallography suggests a pentameric doughnut-shaped structure for the B subunit bound to lipid, the outer ring having a diameter of 60 Angstroms and the inner ring 20 Angstroms (Ludwig et al., 1986).

The A subunit consists of two polypeptide chains initially secreted as a single polypeptide chain, which is nicked by bacterial proteases after secretion of the cholera toxin (Gill and Rappaport, 1979; Mekalanos et al., 1979). The two chains A_1 (22 000 Da) and A_2 (7 000 Da) are linked by a disulphide bond, and are non-covalently associated with the B subunits (Gill, 1976; van Heyningen, 1976, 1977; Ribi et al., 1988). (See fig. 1.1 for a diagrammatic representation of the cholera toxin molecule).

Each subunit plays a different role in the action of the toxin. Van Heyningen (1976) showed that the function of the B subunit was to bind to the cell membrane receptor and that the A subunit possessed the toxic enzymic activity of the toxin. This explained an earlier observation that choleragenoid, the naturally occuring aggregate of B would protect cells (by competing for the receptor) and even intact loops of gut (Pierce, 1973) from subsequent challenge with toxin (Holmgren et al., 1974; Gill and King, 1975).

The activity of subunit A was not affected by ganglioside preincubation, and all the activity was found specifically in the A_1 peptide (van Heyningen, 1976). This indicated that the A subunit played no role in the binding of the toxin to the cell surface receptor.

The B subunit has been completely sequenced (Nakashima et al., 1976; Lai et al., 1977) and the first forty residues show a significant similarity to the epsilon chains (which bind to the cell receptor) of glycoprotein hormones such as thyrotropin, luteinizing hormone, human chorionic gonadotropin and follicle stimulating hormone (Ledley et al., 1976; Kurosky et al., 1977). A secondary structure prediction has been made for subunit B (Duffy and Lai, 1979) based on the method of Chou and Fasman (1978). The molecule shows as much as 35% helical content. The A subunit has also been fully sequenced at the DNA level (Mekalanos et al., 1983) a partial amino acid sequence is known (Jacobs et al., 1974; Lai et al., 1979; Duffy et al., 1981).

1.1.2 Mechanism of Toxicity

Unlike other bacterial toxins such as diphtheria toxin (Uchida, 1982) cholera toxin is not cytotoxic, yet it has profound effects on intestinal cells, resulting in a massive efflux of electrolytes which is indirectly responsible for the symptoms of cholera almost certainly due to an elevation of cyclic AMP from activated adenylate cyclase (Lai, 1980).

The toxin also has effects on other cell types. Glycogenolysis is promoted by the toxin in liver cells and platelets (Zieve et al., 1971). The rate of lipolysis has been shown to be proportional to the amount of toxin added to fat cells (Greenhough et al., 1970; Cuatrecasas, 1973c). Most of these effects are attributable to raised levels of cyclic AMP.

However, more recent studies have shown that some of the toxin's effects appear to be mediated by cAMP independent mechanisms (Aranda and Samuels, 1984; Imboden et al., 1986). Cholera toxin was shown to decrease thyroid hormone nuclear receptors in cultured GH_1 cells, in a time and dose-dependant fashion. The decrease in the number of receptors did not correlate with the amount of cAMP induced by the toxin. Furthermore, in the same cells forskolin, which is able to increase cAMP levels up to 500-fold, had no effect on the number of nuclear thyroid hormone receptors. Aranda and Samuels (1984) proposed that the toxin was somehow decreasing receptor synthesis by a cAMP independent mechanism.

Imboden (1986) showed that exposure of the malignant human T-cell line Jurkat to cholera toxin for three hours reduced the antigen receptor number on the cells. This effect could not be mimicked by the B subunit alone (indicating that mere binding was not enough to produce the effect) or by incubating the cells with reagents to increase cAMP levels.

There are believed to be three basic steps in the toxic action of the cholera toxin molecule. These are;

1. Binding of the toxin to the cell surface.
2. Internalisation of part or all of the toxin molecule.
3. Activation of the adenylate cyclase enzyme.

In order to examine each step of the mechanism in detail, they will be described separately. Fig. 1.2 gives a summary of the steps of intoxication by cholera toxin.

1.1.2.1 Binding of the Toxin to the Cell Surface

There is little doubt that ganglioside GM_1 (see section 1.2.4) is the major receptor for cholera toxin on the cell surface (Cuatrecasas, 1973b; Holmgren et al., 1975; Moss et al., 1976b; Reed et al., 1980). There is a strong specificity of cholera toxin for GM_1 ganglioside; other gangliosides bind very much less tightly and do not inhibit the binding of the toxin to the cell surface to the same extent (King and van Heyningen, 1973; Staerk et al., 1974).

Cholera toxin has therefore been used to locate and quantify GM_1 ganglioside on different cells (Hansson et al., 1977; Ackerman et al., 1980). The binding of the toxin to GM_1 is tight and irreversible with a dissociation constant of about 10^{-9}M (van Heyningen, 1983).

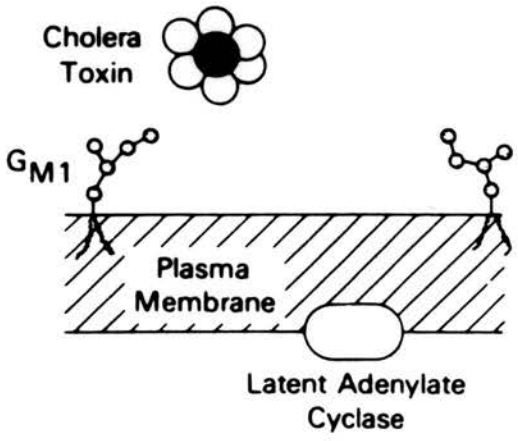
Nevertheless, it has been suggested that glycoproteins play a role in the binding of cholera toxin to intestinal cells (Morita et al., 1980). However, the evidence provided for glycoprotein binding was shown on a fairly artificial system. Glycoproteins were extracted by lectin affinity chromatography and run on SDS Page gels which were overlaid by iodinated cholera toxin. Bound toxin was located by autoradiography. One cannot be sure that the glycoproteins had the same conformation in situ and that they were available for toxin binding in the membrane. In fact the authors state that the presence of these glycoproteins alone is not sufficient evidence to say they are involved in the mechanistic action of cholera toxin.

Fig.1.2

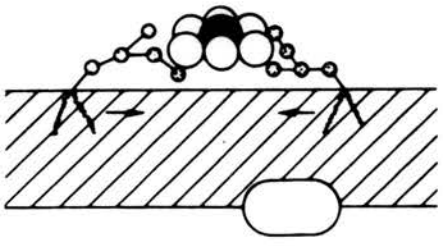
Summary of the Mechanism of Action of Cholera Toxin.

Taken from Fishman and Brady, 1976.

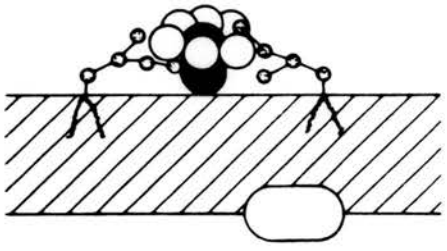
1) Approach



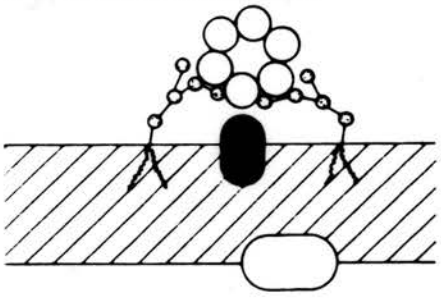
2) Binding



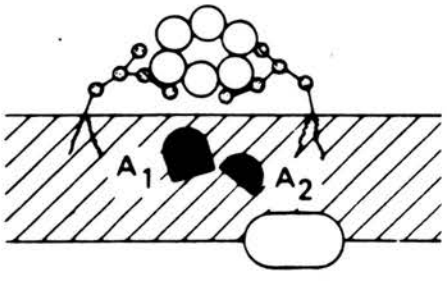
3) Conformational Change



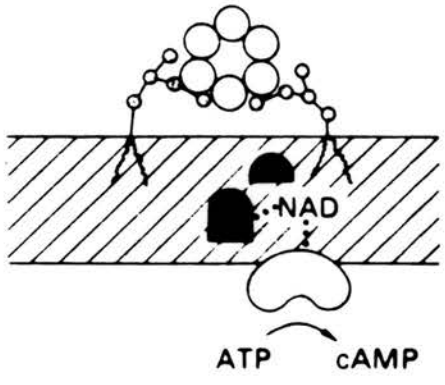
4) Dissociation and Entry



5) Penetration and "Activation"
of A Subunit



6) Activation of Cyclase



Extensive studies involving direct extraction of the receptor from the membranes did not confirm a possible glycoprotein receptor (Critchley et al., 1981). No further evidence for a glycoprotein receptor has emerged recently.

The interesting phenomenon of patching (grouping of cell surface molecules into patches which does not require cellular energy) and capping (accumulation of cell surface molecules at one pole of a cell which requires cell metabolic energy) of either cholera toxin and/or GM_1 to which it binds has been observed only on lymphocytes (Revesz and Greaves, 1975; Kellie et al., 1983; Speigel et al., 1984). The B subunit alone also induced capping (Craig and Cuatrecasas, 1976; Sedlacek et al., 1976). The redistribution of the surface bound cholera toxin or inserted gangliosides to which cholera toxin had bound was observed at 37°C and was blocked by drugs which interfere with the cytoskeleton. These results suggested that GM_1 might be associated with a membrane-spanning protein which interacted with the cytoskeleton.

GM_1 ganglioside itself cannot interact with the cytoskeleton as it is too short, spanning only the upper leaflet of the bilayer. Further support for the idea that GM_1 is associated, probably with a protein linked to the cytoskeleton, has come from detergent extraction experiments, which indicated that cholera toxin-GM_1 complexes remain associated with the cytoskeleton under conditions which normally remove the majority of plasma membrane lipids (Sahyoun et al., 1981; Streuli et al., 1981).

It has been shown that multivalent binding of the toxin appears to be essential for toxin action. Fishman and Atikaan (1980) correlated the inhibition of adenylate cyclase activation, on different cell lines by GM_1 oligosaccharide addition with the density of cell receptors for cholera toxin. They concluded that multivalent binding of cholera toxin was required for the adenylate cyclase activation to occur. The lateral redistribution of the toxin that is sometimes observed may occur therefore in order to achieve the full complement of five gangliosides bound to the B subunit ring of a toxin molecule (Bennett and Cuatrecasas, 1976; Craig and Cuatrecasas, 1976; Sahyoun and Cuatrecasas, 1975). Recently Ribi et al. (1988) have shown that cholera toxin on an artificial bilayer binds pentavalently to ganglioside.

1.1.2.1.1 Conformational Change on Binding?

When cholera toxin binds to GM_1 the toxin alters its conformation and direct evidence for such an event has come from different experimental techniques.

The first observation was that a blue shift occurred in the intrinsic protein fluorescence spectrum after the whole toxin or just its B subunit had bound to ganglioside GM_1 (Mullin et al., 1976; Moss et al 1977a) or to just the oligosaccharide portion of the ganglioside (Fishman et al., 1978).

This change in spectrum did not occur with any other ganglioside tested. As intrinsic fluorescence of proteins is due primarily to tryptophan residues, these experiments suggest a possible role of this residue in binding of toxin to ganglioside. This binding role of tryptophan residues is supported by the evidence from De Wolf et al. (1981) that modification of the tryptophan residues with specific reagents prevented binding of the B subunit to ganglioside in addition to the self-aggregation of the B subunit alone.

Nuclear magnetic resonance has also indicated that binding of toxin to the carbohydrate portion of GM_1 produces a change in the environment of the tryptophan residue (Sillerud et al., 1981).

Therefore the conclusions from fluorescence, nuclear magnetic and modification studies implicate a tryptophan residue in binding of the toxin to its receptor and show that the toxin molecule undergoes a conformational change.

In 1982, van Heyningen showed that the conformational change in the B subunit was transferred to the A subunit. In this experiment only A subunit was labelled with Nbf-chloride (a fluorescent reagent) and binding of GM_1 to the toxin produced a change in the fluorescence spectrum of the A subunit which reached a maximum when only one ganglioside was bound per toxin molecule.

The conformational change which occurs in the toxin molecule when it binds to GM_1 is thought to be important in the insertion process of the active A subunit into the cell (see section 1.1.2.2).

1.1.2.2 Internalisation of Part or All of the Toxin Molecule?

There is a time lag of 15-90 min, depending on the type of cell, between binding of the toxin and activation of adenylate cyclase (Cuatrecasas, 1973a; Gill and King, 1975; Bennet and Cuatrecasas, 1975; Fishman, 1980). If broken cells were incubated with toxin, no time lag was observed (van Heyningen and King, 1975), which suggested either the involvement of a membrane internalisation process or it might also represent a surface lateral regrouping of cholera toxin receptors.

Over the last decade four theories have emerged for the internalisation of cholera toxin which could account for this lag phase and they have tried to provide an explanation as to how the active A_1 peptide reaches its site of action, namely the regulatory component of the adenylate cyclase enzyme complex (see section 1.1.2.3).

Gill (1976) hypothesised that the B subunits of the toxin formed a hydrophilic channel, through which the A component could pass. Indeed experiments with lipid bilayers showed that the toxin could induce permeability changes, perhaps indicating the formation of a pore or channel (Moss et al., 1977b; Tosteson and Tosteson, 1978).

In this model the time lag would be the time taken to form the channel and for the A subunit to pass through. However, the above model has been discounted by hydrophobic photolabelling experiments (Tomasi and Montecucco, 1981; Wisnieski and Bramhall, 1981) and X-ray crystallography (Ribi et al., 1988), which show no evidence for penetration of the membrane by the B subunits.

Another theory has been that cholera toxin undergoes receptor mediated endocytocis, with processing in the lysosomes allowing dissociation of the active subunit (Lin and Taniuchi, 1980; Houslay and Elliot, 1981; Janicot and Desbuquois, 1987). In this model the time lag would be the time taken for the toxin to be taken up and A_1 peptide to dissociate from the endocytosed vesicle.

The hypothesis by Lin and Taniuchi (1980) was based on the fact that lysosmotropic agents reduced the effects of the toxin on HeLa cells, however in their experiments the toxin concentration was high and the concentrations of chloroquine and methylamine used were greater than the amounts needed to inhibit diphtheria toxin which is now believed to use receptor-mediated endocytocis as its mode of entry (Olsnes and Pihl, 1982). In 1981 Gill et al. found that concentrations sufficient to inhibit receptor mediated endocytosis had no effect on the action of cholera toxin on Chinese hamster ovary cells. Therefore this mode of entry might be non specific and occur only on account of the high toxin concentration used.

Fishman has studied the effects of lysosmotropic agents on choleragen action fairly extensively and he states that their mechanism of action is not clear (Fishman, 1982). Other workers have shown that these agents also appear to interfere with ligand receptor clustering and internalisation (Fitzgerald et al., 1980; Pastan and Willingham, 1981).

The theory that is becoming most widely accepted is that of A_1 peptide penetration or translocation across the membrane (Fishman and Brady, 1976). Dissociation of the A_1 peptide from the whole toxin and its subsequent internalisation might account for the observed time lag. Binding of the toxin molecule would induce a conformational change in the molecule (Fishman et al., 1978), so allowing penetration of the active A_1 peptide into the membrane thus perturbing it (Moss et al., 1976a; 1977b; Tosteson and Tosteson, 1978). Persuasive experimental evidence has come from the hydrophobic photolabelling experiments of Tomasi and Montecucco (1981) and Wisnieski and Bramhall (1981) who showed that the A subunit was photolabelled by a lipid probe inside the membrane after the toxin had bound to the surface. However, it is not clear whether the whole A_1 polypeptide has to penetrate the membrane, as small proteolytic fragments are claimed to show activity (Matuo et al., 1976; van Heyningen and Tait, 1980).

Van Heyningen (1977) reported that cross-linked cholera toxin could activate intact cells suggesting perhaps that the A_1 peptide did not need to dissociate from the whole molecule.

Ward et al. (1981) showed that the A_1 peptide was not hydrophobic; that it might not enter the lipid bilayer. So perhaps the A subunit just penetrates the membrane and its active portion is placed near its site of action at the regulatory subunit of adenylate cyclase.

The simplest theory of internalisation was originally suggested by van Heyningen and King in 1975, based on their observation that subunit A was active alone even with intact cells, although at low specific activity. The high local concentration of toxin after binding GM_1 may result in random entry of a few active toxin molecules, which is all that are needed for maximal activation of cyclase. The time required for dissociation of the A_1 peptide and then its internalisation would account for the observed time lag.

1.1.2.3 Activation of Adenylate Cyclase

The mechanism of activation of the adenylate cyclase enzyme is quite well understood. It was Gill who first reported that NAD^+ is required for cholera toxin activation of adenylate cyclase (Gill, 1975; 1976). Moss et al. (1977a) discovered that cholera toxin catalysed the hydrolysis of NAD^+ to ADP-ribose and nicotinamide and Moss and Vaughan (1977) later showed that the same reaction was stimulated by the presence of D or L arginine or guanidine which acted as acceptors for the ADP-ribose moiety.

In pigeon erythrocytes, when [^{32}-P]-NAD^+ was supplied, cholera toxin catalysed the ADP-ribosylation of specific proteins (Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Gill 1979). The main protein which was affected by the toxin was a 42 000 dalton protein identified as the regulatory protein N (of the adenylate cyclase enzyme) by Pfeuffer in 1977.

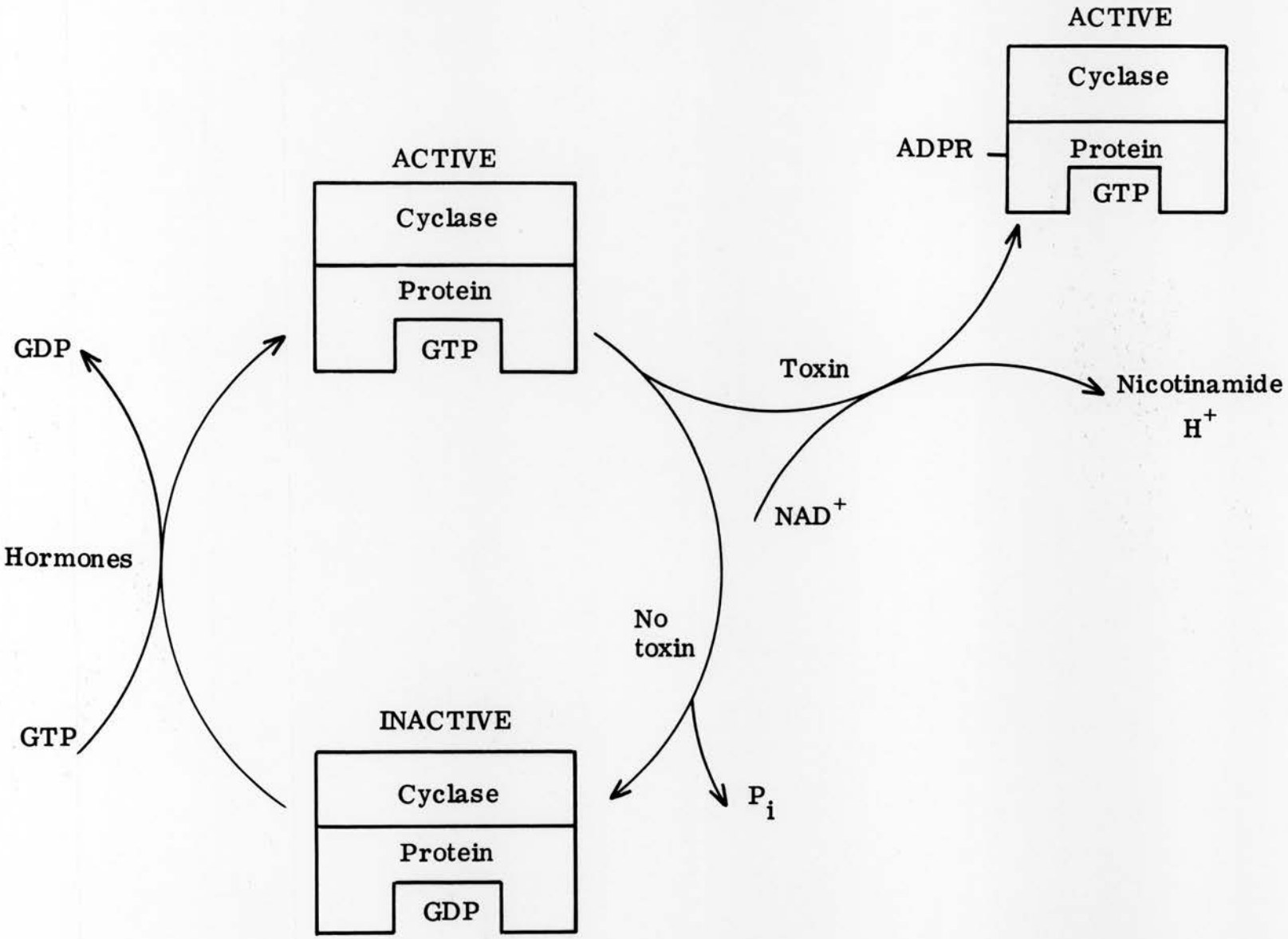
Substantial evidence has emerged to show that the regulatory component of adenylate cyclase (which binds guanine nucleotides) couples the hormone receptor to the catalytic subunit, which synthesises cyclic AMP (reviewed by Ross and Gilman, 1980; Rodbell, 1980).

Cassel et al. (1977) showed that ADP ribosylation of the regulatory protein N_s of adenylate cyclase prevented the GTP bound to it from becoming hydrolysed thus deactivating the enzyme. In effect it maintained the active state of the enzyme.

All the above points may be summarised in a schematic representation of cholera toxin activation of adenylate cyclase (See fig. 1.3).

Fig.1.3

Activation of the Adenylate Cyclase Enzyme by Cholera Toxin. (Reproduced from van Heyningen, 1977).


ACTIVE
Cyclase
Protein
GTP
ADPR
ACTIVE
Cyclase
Protein
GTP
GDP
Toxin
Nicotinamide
H^+
NAD^+
Hormones
No
toxin
GTP
INACTIVE
Cyclase
Protein
GDP
P_i

1.1.3 Other Bacterial Toxins and Plant Toxins

Cholera toxin is very similar to the heat-labile enterotoxin produced by Escherichia coli which produces the milder symptoms of 'travellers diarrhoea' in humans. The two toxins share extensive immunological cross-reactivity (Clements and Finkelstein, 1978; Lindholm et al., 1980). Both toxins have ADP-ribosyltransferase activity and they both irreversibly activate adenylate cyclase through covalent modification of the GTP-binding regulatory subunit (Gill and Richardson, 1980). Furthermore, the two toxins have the same subunit structure (Clements et al., 1980) and show much sequence homology (Mosley and Falkow, 1980; Spicer et al., 1981; Spicer and Noble, 1982; Dykes et al., 1985). There is an indication however, that the two toxins show subtle differences in their binding characteristics, stemming from the observation that E.coli heat-labile toxin has a relatively high affinity for galactose polymers and can be purified on agarose columns, whereas the binding of cholera toxin to these columns is weak (Clements and Finkelstein, 1979; Clements et al., 1980).

Several other bacterial toxins which are responsible for quite different diseases with very different metabolic effects on cells show distinct similarities in their attack on cells. The main similarities may be seen in their structure, their binding to cells and their mode of action.

1.1.3.1 Similarities in Structure

Bacterial toxins have the common problem of approaching cells from the outside and acting on intracellular targets. Hence the toxins have adopted the successful strategy of having a binding component which recognises the cell surface and an active component whose entry into the cell is assisted by the binding component.

Cholera toxin structure has been mentioned, as has that of E.coli heat labile toxin, which is essentially the same. Diptheria toxin secreted by Corynebacterium diphtheriae has an essentially similar structure to cholera toxin having 2 chains (Collier, 1975; Pappenheimer, 1977; Uchida, 1982). It is secreted as a single polypeptide that can be proteolytically cleaved into two chains, fragment A (24 000 Da) and fragment B (38 000 Da) which remain connected by a disulphide bond. The B fragment bonds to an unknown receptor, which might be the anion transporter (Olsnes and Sandvig, 1986), and this allows the active A fragment to enter the cell.

Less is known about the clostridium neurotoxins tetanus secreted by Clostridium tetani and botulinum secreted by Clostridium botulinum.

Both are secreted as single polypeptide chains of about 150 000 Da and both are proteolytically nicked to form two chains of about 100 000 Da and 50 000 Da joined by one or more disulphide bonds. Again, one of the chains, the heavier one, shows binding activity. By analogy to the other bacterial toxins, although no activity is known, the active domain is believed to reside in the light chain (van Heyningen, 1980; Sugiyama, 1980; Mellanby and Green, 1981). The toxin which causes whooping cough, pertussis secreted by Bordetella pertussis has also been shown to have several binding components and an active component, like cholera toxin (Tamura et al., 1982).

The plant toxins, abrin, ricin and modeccin all have both a binding chain and an active chain (Olsnes and Pihl, 1976; 1982).

1.1.3.2 Similarities in Binding

The two major cell surface components implicated in the binding of bacterial toxins have been glycolipids and glycoproteins. These cell surface components are termed receptors, yet they are not 'true' receptors in an endocrinological sense, whereby just the binding of the ligand causes the receptor to initiate the physiological response. Indeed it has also been shown recently that diphtheria toxin remains toxic to cells even if its binding component is replaced by another molecule which recognises the cell surface thereby discounting the need for a specific receptor molecule for the ligand (Johnson et al., 1988).

It has been shown that GM_1 ganglioside is the major receptor for cholera toxin, (see section 2.2.0). E.coli heat-labile toxin has also been shown to bind GM_1 ganglioside (Svennerholm and Holmgren, 1978; Moss et al., 1979,1981). However there is some evidence for a galacto protein receptor for the E. coli toxin (Griffiths et al., 1986), as E. coli toxin cannot compete out cholera toxin binding 100%.

Although, as mentioned previously, it has been shown that the diphtheria toxin receptor is not needed for diphtheria toxin to exert its toxicity on cells, provided the active subunit is attached to another cell surface recognising ligand, there is evidence that the usual receptor might be a protein. This has been reviewed by Eidels et al. (1983). It has been suggested that it is the anion transporter (Olsnes and Sandvig, 1986). Both botulinum and tetanus toxins are believed to bind to a ganglioside molecule, possibly GT_{1b} or GD_{1b} (Holmgren et al., 1980; Kitamura et al., 1980). There is little information on the nature of the receptor for pertussis toxin. However, it may be a molecule containing sialic acid, as the B protomer of the toxin binds to the sialoprotein haptoglobulin (Irons and Maclennan, 1979; Tamura et al., 1982).

Finally, it has been shown that the plant toxin ricin, binds to plasma membrane molecules with terminal galactose residues (Pardoe et al., 1969; Baenziger and Fiete, 1979; Sandvig et al., 1976).

1.1.3.3 Similarities in Mode of Action

To date, at least five bacterial toxins are known to share the same enzymic reaction: catalysis of the cleavage of NAD^+ and ADP-ribosylation of a protein substrate.

Both cholera toxin and E. coli LT toxin act on the GTP-binding regulate subunit which stimulates adenylate cyclase (Gill and Richardson, 1980).

Pertussis toxin, however, acts on the GTP binding regulatory subunit Gi which inactivates adenylate cyclase (Bokoch et al., 1983). The site of action of diphtheria toxin is the protein elongating factor 2 (EF2) which is necessary in protein synthesis and its ADP-ribosylation eventually leads to the death of the cell (Collier, 1975; Pappenheimer, 1977; Uchida, 1982).

The molecular action of the plant toxins is to modify one or two nucleotides on 28s rRNA (Endo et al., 1987) and thus prevent protein synthesis (Olsnes and Pihl, 1982). As yet, no molecular action has been determined for tetanus and botulinum toxins.

It is therefore evident that bacterial and plant toxins have many properties in common. (See Table 1 for a summary of these similarities and differences).

Table 1 Similarities and Differences Between Toxins

Toxin	Mol.wt. of toxin	Active component			Binding component		
		Structure	Mol.wt.	Target	Structure	Mol.wt.	Target
Affecting adenylate cyclase							
Cholera	82000	Subunit A =A1 peptide +A2 peptide	27000 22000 5000	N_s protein of adenylate cyclase	Five B subunits	11600 each	ganglioside GM1
E.coli heat labile	91000	Subunit A =A1 peptide +A2 peptide	30000 25000 5000	N_s protein of adenylate cyclase	Five B subunits	11800 each	ganglioside GM1 and glycoproteins
Pertussis	117000	S-1	28000	N_i protein of adenylate cyclase	S-2 S-3 Two S-4 S-5	23000 22000 11700 9300 each	not known
Affecting protein synthesis							
Diphtheria	62000	A chain	24000	EF2 (dipthamide)	B chain	38000	glycoprotein
Neurotoxins							
Tetanus	150000	L chain	50000	no clear function	H chain	100000	ganglioside GT1b,GD1b
Botulinus	150000	L chain	50000	no clear function	H chain	100000	not known, may be a glycolipid

SECTION TWO

1.2.0 Gangliosides: identification

In 1935, Professor E. Klenk of Cologne University found a type of glycosphingolipid containing an unknown acidic sugar (sialic acid), in the brain tissue of children suffering from infantile amaurotic idiocy (Tay Sachs disease). These lipids had been detected earlier by Landsteiner and Levene (1925) and Walz (1927 a, b) by their reaction with p-dimethylaminobenzaldehyde and orcinol to form a coloured complex. In 1942, Klenk named this type of glycosphingo-lipid, 'ganglioside', as he thought that it was located in ganglion cells. However, in 1951, Yamakawa and Suzuki discovered that ganglioside also occurred in extraneural tissue and fluids.

Gangliosides are the most complex of brain lipids. They are a group of glycosphingolipids characterised by the presence of N-acetyl or N-glycolyl-D neuraminic acid and a variety of other sugars, e.g. glucose, galactose and N-acetyl galactosamine (Kuhn and Wiegandt, 1963). At least 40 different ganglioside structures have been established during the last twenty years (Hakkinen and Kulonen, 1963; Svennerholm, 1963; Tettamenti et al., 1964; Hakamori, 1966; Ando and Yu, 1979) and this number approaches 60 when variation in sialic acid is taken into account (Ledeen, 1983). In addition to variation in type of ganglioside, large differences occur in ganglioside concentrations for different tissues (Ledeen and Yu, 1982).

1.2.1 Structure

Gangliosides are compounds that are hydrophilic at one end of the molecule and hydrophobic at the other. They also have a strong negative charge, due to the presence of one or more sialic acid residue. The hydrophobic end of the molecule consists of two long chain fatty acids which are linked to the amino alcohol sphingosine. The hydrophilic end is mainly carbohydrate, consisting of molecules of hexoses, N-acetyl hexosamines and sialic acid residues. A more detailed description of ganglioside structure may be obtained from the review by Ledeen and Yu, 1982.

The nomenclature of gangliosides is based primarily on the number of sialic acid residues and the carbohydrates which form the oligo-saccharide portion of the molecule. Therefore the letter G stands or ganglioside. M denotes monosialo-one sialic acid; D = disialo-two sialic acids; and T = trisialo-three sialic acids etc. The numbers written as a subscript stand for; 1, the major neutral tetrasaccharide chain, 2, the chain lacking the terminal galactose and 3, for the chain lacking galactosyl-N-galactosamine (Svennerholm, 1970).

Thus, the majority of mammalian gangliosides are based upon GM_1 as the basic structural unit (Ledeen and Yu, 1982). This unit is ceramide glucose-galactose, (sialic acid attached) - N-acetyl galactosamine-galactose (See fig. 1.4).

The ceramide portion is believed to be inserted in the upper leaflet of the lipid bilayer and the oligosaccharide head is exposed (Hakamori, 1981).

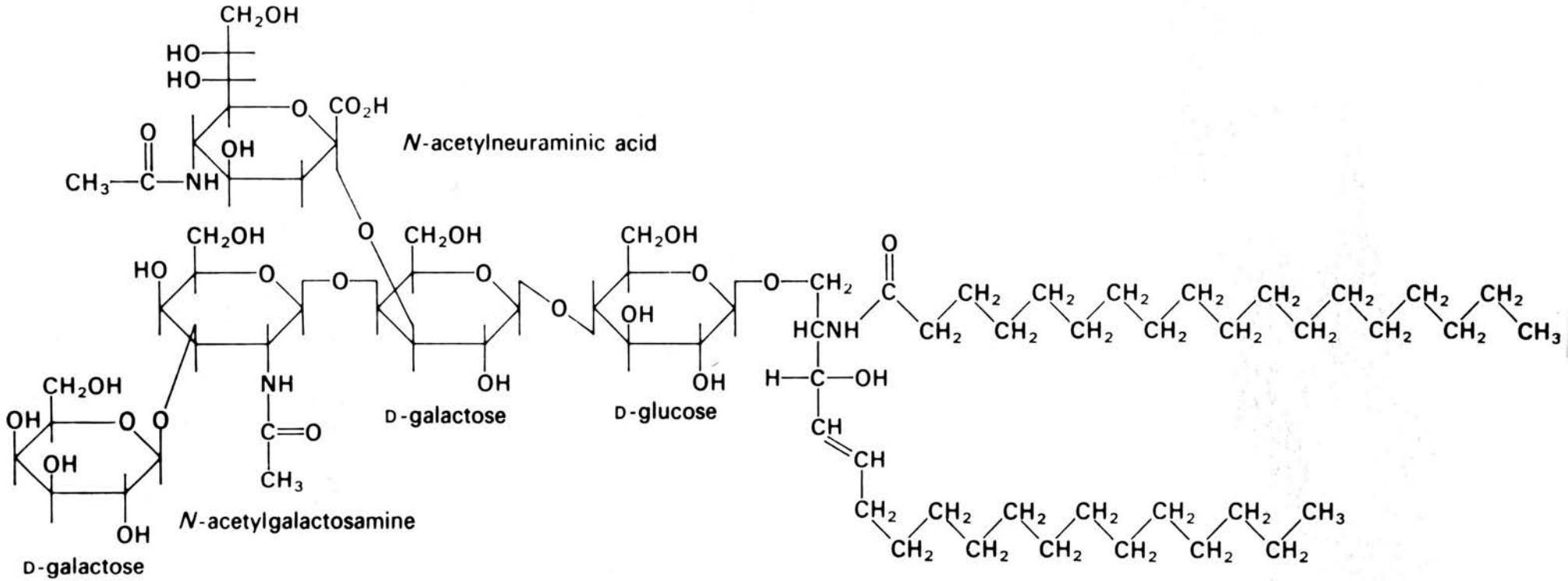
1.2.2 Biosynthesis

The biosynthesis of gangliosides will be covered very briefly, dealing only with the hydrophilic carbohydrate portion of the structure and not the ceramide as it is the oligosaccharide end of the molecule which appears to interact with the bacterial protein cholera toxin.

Addition of monosaccharides to the ceramide is catalysed by glycosyltransferases. Each reaction involves the transfer of a sugar residue from a donor to an acceptor. The addition is ordered and each glycosyltransferase is highly specific. The pathway for the biosynthesis of the major gangliosides has been worked out in detail (Roseman, 1970; Fishman and Brady, 1976; Basu et al., 1976, 1979). See fig. 1.5 for an outline of this biosynthetic pathway for the major mammalian gangliosides.

Fig.1.4

Structure of GM_1 ganglioside
(Reproduced from van Heyningen, 1977)


N-acetylneuraminic acid
D-galactose
D-glucose
N-acetylgalactosamine
D-galactose
Hydrophilic portion
sugars
Hydrophobic portion
ceramide

1.2.2.1 Cellular Localisation of Glycosyltransferase

The localisation of glycosyltransferases within cells is a little controversial. Keenan et al. (1974) demonstrated that in human liver, these enzymes are associated with the golgi apparatus. In cultured cells, it has been shown that there is some enzyme activity in the plasma membrane (Reviewed by Shur and Roth, 1975).

1.2.2.2 Cellular Distribution of Gangliosides

It was originally assumed that all gangliosides in non-neuronal cells were located in the plasma membrane (see review by Stoffel, 1971). However, Keenan and co workers showed that for rat liver and bovine mammary gland, only 10 to 25% of the total cellular gangliosides were located in the plasma membrane, the rest being distributed amongst the intracellular membranes (Keenan et al., 1972 a, b). When the biosynthesis of gangliosides in cultured cells was examined, most of the gangliosides were found on the cell surface, with some in lysosomes, others at the golgi, and the rest being in transit from the golgi to the plasma membrane (Miller-Prodraza et al., 1982).

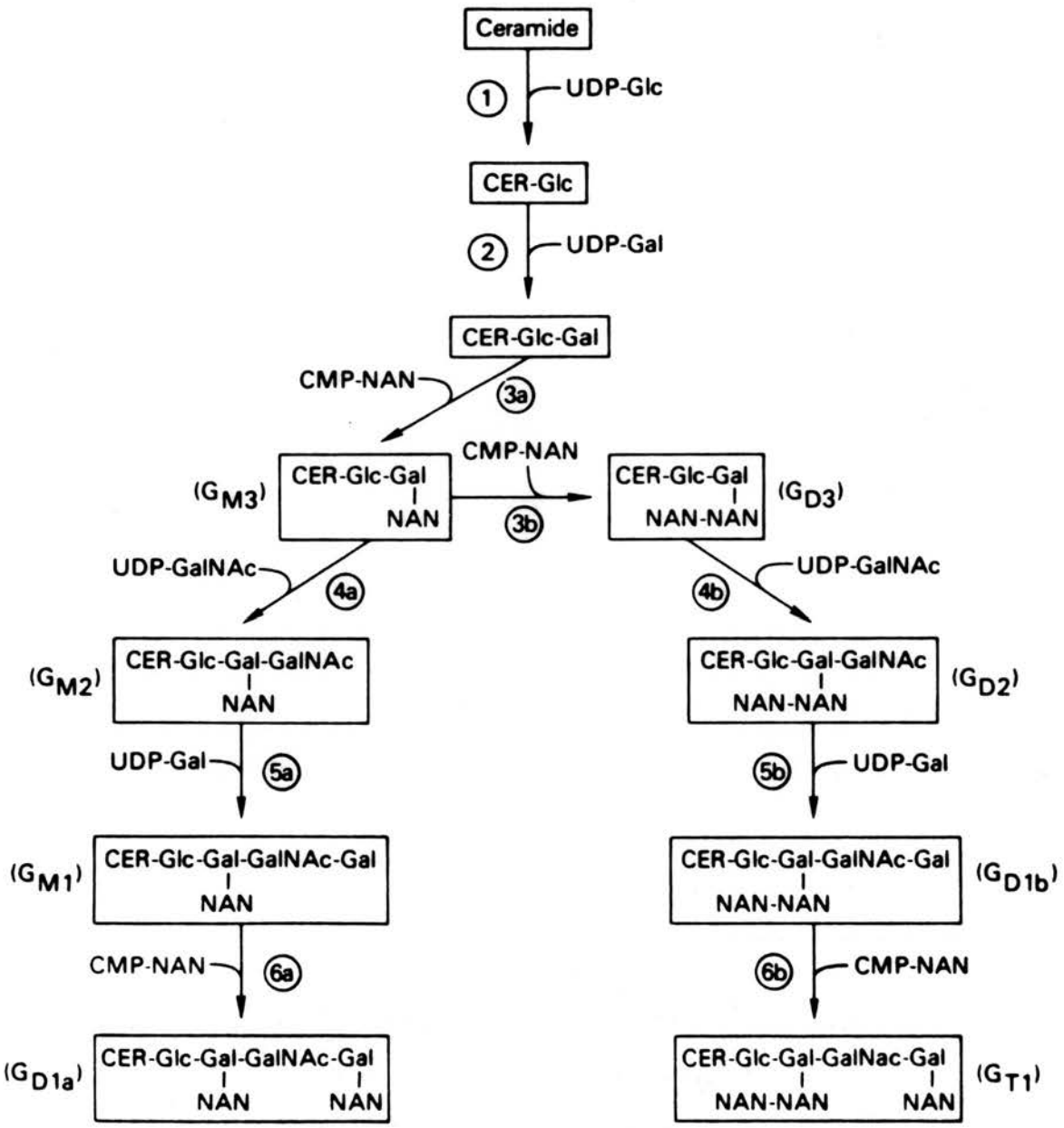
More recently, Matyas and Morre (1987) have extended the original work by Keenan et al., (1972) and specified the individual gangliosides in highly purified membrane fractions from rat liver. They found that individual gangliosides were non-homogeneously distributed and that each membrane fraction could be characterised by a unique ganglioside composition.

The membranes they examined were plasma, nuclear, golgi and endoplasmic reticulum.

Spiegel et al. (1988) found an asymmetric distribution of gangliosides in rat renal brush-border and basolateral membranes and suggested that this indicated a specific sorting mechanism for epithelial plasma membrane glycolipids, and that this might be linked to their possible functional roles in each of the membranes. Thus, relatively little information has been accumulated about the localisation of ganglioside molecules within cells. It is becoming evident that the plasma membrane is not the only site of localisation within the cell, but it is usually the predominant one.

Fig.1.5

Summary of the major pathway of carbohydrate addition in ganglioside biosynthesis.
(Reproduced from Fishman and Brady, 1976)


Ceramide
1
UDP-Glc
CER-Glc
2
UDP-Gal
CER-Glc-Gal
CMP-NAN
3a
(GM3)
CER-Glc-Gal
NAN
CMP-NAN
3b
CER-Glc-Gal
NAN-NAN
(GD3)
UDP-GalNAc
4a
UDP-GalNAc
4b
(GM2)
CER-Glc-Gal-GalNAc
NAN
CER-Glc-Gal-GalNAc
NAN-NAN
(GD2)
UDP-Gal
5a
5b
UDP-Gal
(GM1)
CER-Glc-Gal-GalNAc-Gal
NAN
CER-Glc-Gal-GalNAc-Gal
NAN-NAN
(GD1b)
CMP-NAN
6a
6b
CMP-NAN
(GD1a)
CER-Glc-Gal-GalNAc-Gal
NAN
NAN
CER-Glc-Gal-GalNac-Gal
NAN-NAN
NAN
(GT1)

1.2.3 Functions

Gangliosides have been implicated in a variety of cellular phenomena but their exact physiological role remains unknown. The large variety of glycosphingolipid types suggest they are well suited to interact with exogenous ligands or regulate protein receptor function, due to their carbohydrate being exposed and their lipid being inserted within the membrane (Nagai and Iwamori, 1980). Structural rigidity might be conferred on the membrane by the high content of ganglioside present within the bilayer.

1.2.3.1 Structural Rigidity of Membranes

It has been shown using the sensitive techniques of nuclear magnetic resonance and electron spin resonance spectroscopy that liposomes containing sphingolipids are more rigid than those with just phospholipids (Abrahamsson et al., 1977; Sharom and Grant, 1977). An explanation of this was given by Yamakawa and Nagai in 1978, who suggested that glycosphingolipids had both hydrogen acceptors and a variety of hydrogen donors, so they could make stable hydrogen bond interactions and have a more rigid and ordered region in membranes than phospholipids which had only hydrogen acceptors and thus could not undergo the same interaction.

1.2.3.2 Cell Differentiation and Growth Markers

Ganglioside patterns and quantities alter considerably during development of the brain (Suzuki, 1965; Dreyfus et al., 1975). Recently Facci et al., (1988) demonstrated that both endogenous and exogenous gangliosides could influence the state of differentiation of astroglial cells, *in vitro.*

Transformed cells lack the growth restraints in culture which arise as a result of cell contact or density changes. They have a reduced requirement for nutrients and they have a different morphology to normal cells. As a most striking change occurs in their ganglioside pattern and synthesis, it is thought that the alteration in growth characteristics of the transformed cells may be related to this change in gangliosides (Brady and Fishman, 1974; Hakamori, 1975). Indeed some gangliosides such as GD_{1b} have been found to be important specific oncogenic markers (Feizi, 1985).

The role of endogenous and exogenously added gangliosides is being increasingly studied (Alessandri et al., 1987; Spiegel and Fishman, 1987, Hanai et al., 1987). The work by Spiegel suggests a possible modulatory role of gangliosides on a signal transduction mechanism activated by exogenously applied growth factors.

1.2.3.3 Interaction with Biologically Active Factors

Gangliosides have been implicated as receptors for a wide range of biologically active factors, which will be dealt with very briefly here.

The term receptor here is only used to indicate that gangliosides have been shown to have specific binding interactions with certain ligands. GM_1 is the receptor for cholera toxin (see Section 1.2.4). Gangliosides have also been implicated as receptors for other bacterial toxins (see Section 1.1.3.2). They have also been shown to interact with glycoprotein hormones and viruses. These interactions have been reviewed by Hakomori, 1981.

1.2.4.0 Evidence that GM_1 is the Receptor for Cholera Toxin

In 1971, van Heyningen and co workers showed that by preincubating cholera toxin with a crude ganglioside mixture they could prevent cholera toxin binding to cells. Two years later, three independent studies first recognised GM_1 as the probable cell surface receptor for cholera toxin (Holmgren et al., 1973; Cuatrecasas, 1973b; King and van Heyningen, 1973). Holmgren's group showed that GM_1 inhibited the effects of cholera toxin down to equimolar concentrations. They also showed that two other glycolipids ie. GD_{1a} and GA_1 reacted with the toxin yet they only inhibited cholera toxin when at 500 times greater concentration than GM_1. The same group also showed that only GM_1 gave rise to a precipitation band, in an Ouchterlony-type (Ouchterlony, 1958) double diffusion test, giving direct evidence for specific binding between the multivalent toxin and GM_1 (probably in micelles) in a system independent of biological assays.

Studies with various cell types, including small intestinal mucosal cells from different species, demonstrated a direct relationship between the cell content of GM_1 and the number of toxin molecules the cell could bind (Holmgren et al., 1975; Hansson et al., 1977).

Mullin et al. (1976) observed that pretreatment of cell membranes with cholera toxin specifically blocked the membrane GM_1 ganglioside from reacting with galactose oxidase. Exposure of several cell lines to sodium butyrate (an agent which induces differentiation in cells) increased the amount of GM_1 in the membranes, with a concomitant increase in the number of toxin receptors (Fishman and Atikaan, 1979).

A very strong support for the receptor role of GM_1 comes from experiments showing that exogenous GM_1 can be incorporated into the cell membrane and then act as a functional receptor. This was first demonstrated by Cuatrecasas (1973b) who observed increased binding capacity and lipolytic responsiveness of fat cells which had been exposed to GM_1.

Moss et al. (1976b) added [^{3}H]-GM_1 to a cell line (NCTC 2071) grown in a chemically defined medium without serum (as serum contains trace amounts of ganglioside). Normally these cells lack any detectable GM_1 but they do have an adenylate cyclase enzyme so only broken cell preparations can respond to cholera toxin.

In this experiment, the intact cells took up the ganglioside and exhibited a response to cholera toxin, which was maximal when 100 000 molecules of GM_1 had been incorporated per cell. These cells could also take up other gangliosides, but were not sensitised to cholera toxin when they did (Fishman et al., 1976, Fishman, 1982).

Several other studies have also shown that exogenously added gangliosides, especially GM_1, can stably insert into the plasma membrane and behave as a functional receptor for cholera toxin and are then metabolised by the cell (Spiegel et al., 1983; Fishman et al., 1983).

Spiegel showed that even fluorescent derivatives of GM_1 ganglioside can stably insert into a cell membrane (glioma cell line lacking toxin receptors) and act as a toxin receptor, so, increasing the capacity of the cell to bind toxin and also increasing the level of activation of adenylate cyclase (Speigel, 1985). Therefore, fluorescent ganglioside derivatives have become very important tools in the study of ganglioside function in cells.

Several other lines of evidence support GM_1 as the receptor for cholera toxin. All toxin binding activity on cells and membranes which had been exhaustively delipidated, was removed (Cuatrecasas, 1973a, Fishman and Atikaan, 1979; Critchley et al., 1981).

A protein receptor does not appear to be implicated in cholera toxin binding as proteases do not affect it (Cuatrecasas, 1973a; Fishman and Atikaan, 1979; Critchley et al., 1981). Critchley, 1981 showed very clearly with intestinal lipid extracts separated on thin-layer silica gel and overlayed with ^{125}I-labelled cholera toxin, that the toxin bound only to material corresponding to GM_1. He also showed that if intestinal membranes were dissolved in SDS and separated by SDS-polyacrylamide gel electrophoresis, overlaying the gel with toxin produced a bound toxin band at the front of the gel where the lipids migrated. Therefore it is very evident that there is strong experimental support for GM_1 being the cell surface receptor for cholera toxin (cited in section 1.1.2.1).

SECTION 3

1.3.0 Objectives of the Present Study

The broad objective at the beginning of this work was to 'study some aspects of the interaction between gangliosides and protein ligands in cell membranes'.

The collaboration between Dr. Simon van Heyningen who has many years experience studying bacterial protein toxins and Dr. Peter Garland who is interested in biophysical measuring techniques applicable to cell biology, meant that the expertise for such a study was available.

Dr. Peter Garland was established at Unilever's Colworth Laboratories where he had set up the instrumentation to carry out Fluorescence Recovery After Photobleaching (FRAP) measurements which define the movement of ligands and/or receptors on cell membranes. There was also expertise in Colloidal Gold Electron Microscopy at Colworth.

As cholera toxin was commercially available and is the most understood bacterial protein toxin, we decided to begin the interaction studies using this ligand.

Two lines of study were initiated; one to measure the lateral mobility of ganglioside GM_1 (the cell receptor for cholera toxin) in the plasma membrane and also that of the toxin receptor complex, the second to probe for possible ganglioside sites intracellularly using the technique of colloidal gold immunoelectron microscopy, exploiting the strong specificity of cholera toxin for GM_1 ganglioside.

Technically, both these studies proved to be difficult. The FRAP measurements depended upon the viability of the cells and how well they had been labelled with either fluorescent GM_1 ganglioside or fluorescent cholera toxin. The localisation of possible GM_1 sites intracellularly, persistently showed intense labelling of cell nuclei, which after many months and many controls was taken as a valid though currently unexplainable result (ganglioside GM_1 is not thought to be present within cell nuclei). Therefore the latter result was pursued using biochemical techniques to show the existence of a cholera toxin binding site within a cell nucleus.

Consequently, no direct link was established between the FRAP work and that of electron microscopy and this thesis presents these two studies as separate chapters containing their own introduction, specialised technical details and discussion. Other technical methods which are generally applicable are described in Chapter two. Chapter five contains a description of the preliminary binding studies which were initiated from the electron microscopical determination of a cholera toxin binding site in the nucleus, which is still uncharacterized. Chapter six provides a brief summary of findings and suggests further work which could follow this study.

Chapter 2

GENERAL MATERIALS AND METHODS

Chapter 2

GENERAL MATERIALS AND METHODS

SECTION ONE:

2.1.0 General Materials

The reagents used for experiments mentioned in this chapter were obtained from the following sources:

Cholera toxin	Swiss Serum Institute, Berne, Switzerland Sigma, Poole, Dorset (supplied in lyophilised 1mg batches)
GM_1 ganglioside	Prepared by Mr. N. Gascoyne (see King et al., 1976) by passing mixed brain gangliosides down a column of silica gel Gi to produce pure GM_1 (stored lyophilised at $4^{o}C$).
Fluorescein thiosemicarbazide	Molecular Probes Inc., Junction City, USA
Radioactive iodide (0.5mCi)	Amersham International, Little Chalfont, England
DE52 Sepharose	Pharmacia (Fine Chemicals), Milton Keynes, England
Biogel P-6DG	Biorad Laboratories, Watford, England
Rabbit anti-(cholera toxin) antiserum	Gift from Dr. S. van Heyningen (van Heyningen, 1976)
FITC-labelled goat-anti (rabbit IgG) antiserum	Miles Scientific, Slough, England
Polyclonal anti-GM_1 antibody	Gift from Dr. N. Gregson, Guys Hospital, London
Monoclonal anti-A subunit (cholera toxin) antisera	Gift from Dr. R. Holmes (Holmes and Twiddy, 1983)

Polyclonal anti-A subunit (cholera toxin) (B09) and anti-toxin antisera (A24)	Gift from Dr. R. Holmes (Holmes and Twiddy, 1983)
HPTLC pre coated silica gel 60 glass-backed plates (10cm x 10cm)	E, Merck, Darmstadt, Germany
Sephadex G-25M PD-10 columns	Pharmacia (Fine Chemicals), Milton Keynes, England
Mouse NIH 3T3 fibroblasts	Cell line supplied by Unilever Research Laboratory
Tissue culture medium (DMEM) England	Flow Laboratories, Rickmansworth,
Foetal Calf Serum	Gibco Ltd., Paisley, Scotland
1 x Trypsin/EDTA solution	Gibco Ltd., Paisley, Scotland

The following phosphate buffered saline buffer was routinely used, it is commonly known as Dulbeccos PBS but is referred to as PBS in the text. The buffer contains (140mM NaCl, 3mM KCl, 8mM Na_2HPO^4 and 1.5mM KH_2PO_4) and has a pH of 7.5.

In order to specifically stain gangliosides the Resorcinol reagent was used. It contains 10ml of 2% aqueous resorcinol solution and 0.25ml of a 0.1M CU_2SO_4 solution together with 80ml of 10M HCl. This is made up to a total volume of 100ml with distilled water and stored at room temperature for four hours and then at 4^oC for no longer than a month.

All other reagents were analytical grade and were obtained from Sigma or BDH.

Specialised equipment and materials used for Fluorescence Recovery After Photobleaching (FRAP) or immunoelectron microscopy are described in Chapters 3 and 4 respectively.

SECTION TWO:

2.2.0 General Methods

2.2.1 Cell Culture

Established cell lines of National Institute of Health (NIH) 3T3 mouse fibroblasts were maintained in culture flasks (70 cm^2) (or on coverslips 6cm x 3cm, in petri dishes) in Dulbecco's Modified Eagles Medium (Dulbecco and Freeman, 1959) containing 10% foetal calf serum. The medium was replaced weekly and the cells were trypsinized and 're-seeded' alternatively to prevent them growing to confluency. Cell density was calculated using a Coulter counter.

2.2.2 Preparation of Fluorescent Cholera Toxin

Fluorescent toxin (labelled with fluorescein or rhodamine isothiocyanate) was prepared following an adapted procedure normally used to label antibodies fluorescently (Wood et al., 1965; Goding, 1983).

Freeze-dried cholera toxin (1 mg) was dissolved in 1ml of 0.1M bicarbonate buffer (0.1M sodium carbonate, 0.1M sodium bicarbonate pH 9.5) for several hours. Then 100ug of a suitable fluorochrome eg. fluorescein isothiocyanate (diluted in dimethylsulphoxide 10mg/ml) was added for every mg of cholera toxin and the mixture was incubated in the dark at room temperature for 2 hr.

Unconjugated fluorochrome was removed from the conjugated protein by passing up to 2ml of the mixture down a Sephadex G-25 column (height 5cm, volume 9ml) which had been previously equilibrated with 0.05M Tris-HCl buffer (containing 0.2M NaCl and 1mM EDTA pH 7.5).
The same buffer was used to elute the column and 500ul or 1ml fractions were collected into Eppendorf tubes. The absorbance of the fractions at 280nm (protein) and 495/554nm (fluorescent conjugate) was measured on a Cecil UV spectrophotometer to determine the protein content and the fluorochrome content (fluorescein 495nm; rhodamine 554nm). (See fig. 2.1).

2.2.2.1 Calculation of Fluorescein/Protein Ratio

The fractions containing conjugated protein were pooled and the final absorbance readings taken (see previous section). The molar ratio of fluorescein to protein in the conjugate was calculated, based on a formula proposed by The and Feltkamp (1970a,b) for the labelling of immunoglobulin by FITC. The molar concentrations were calculated using the molar extinction coefficients for both fluorescein and cholera toxin. As 31% of the absorbance at 280nm is contributed to by the fluorescein, this is allowed for in the calculation.

Molar ratio

FLUORESCEIN/CHOLERA TOXIN

$$\left[\frac{A495}{E495}\right] \Big/ \left[\frac{A280-[0.31X\ A495]}{E280}\right]$$

A 495 : Absorbance of fluorescein

E 495 : Extinction coefficient (molar) of fluorescein

A 280 : Absorbance of cholera toxin

E 280 : Extinction coefficient (molar) of cholera toxin

E 495 fluorescein = 73 000 $M^{-1}cm^{-1}$ (Schreiber and Haimovich, 1983)

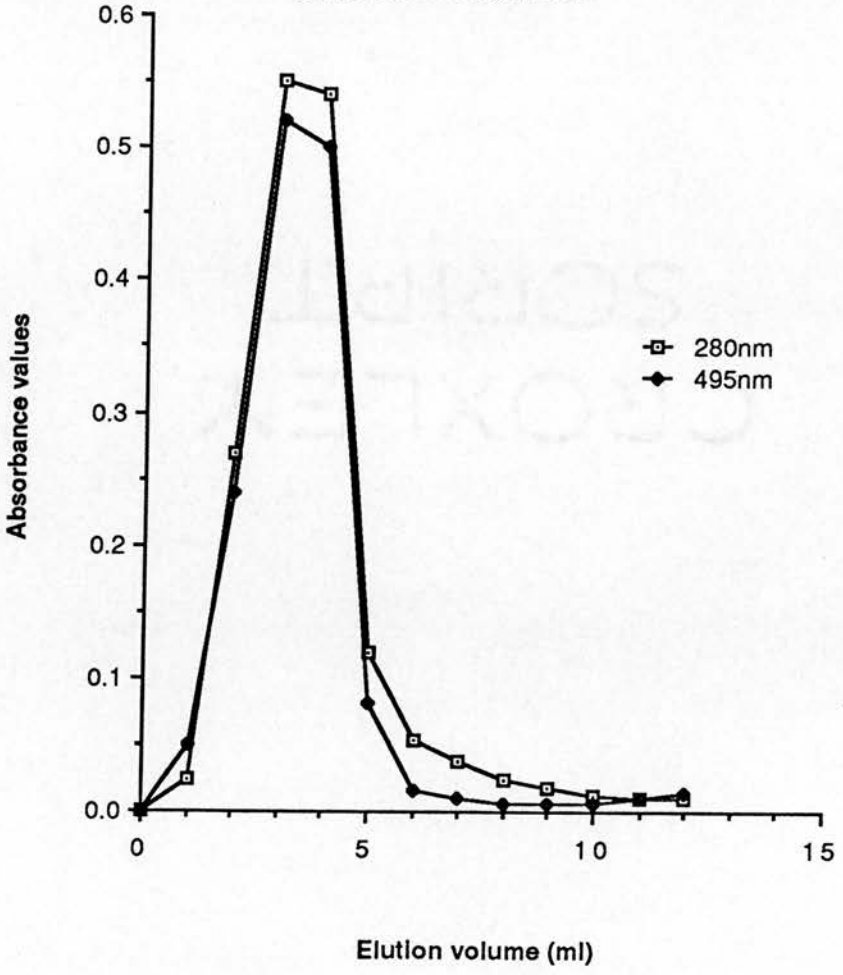
E 280 cholera toxin = 95 760 $M^{-1}cm^{-1}$ (LoSpalluto and Finkelstein,1972)

E 578 rhodamine = 80 000 $M^{-1}cm^{-1}$ (Haughland, 1989)

A ratio of 2 was usually obtained.

The conjugated protein was electrophoresed on a 12% SDS PAGE gel (see section 2.3) to check whether both subunits were labelled, see fig. 2.2. It was also checked by the Ouchterlony Technique (see section 2.11) to see if it still bound to GM_1 ganglioside. It formed a fluorescent precipitation line with the GM_1.

FIG **2.1** **Elution of Fluorescent Cholera Toxin on a G-25 Column**



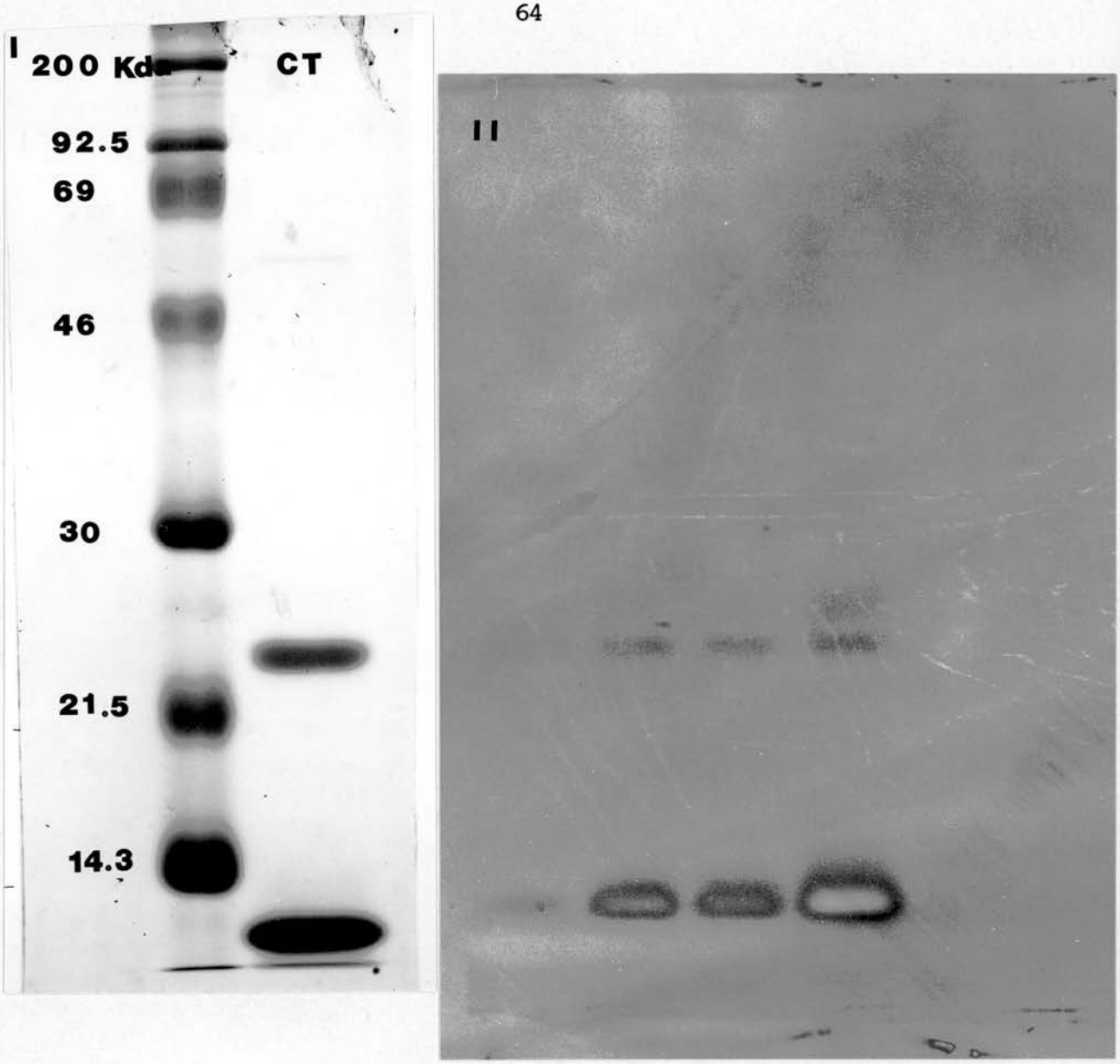


Fig. 2.2 I Coomassie-stained half of gel showing molecular weight markers and the two subunits of cholera toxin.

II UV illuminated other half of above gel showing that both subunits of cholera toxin are fluorescently labelled. Four tracks represent four different preparations.

2.2.3 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was always carried out in the presence of 0.1% w/v SDS (SDS PAGE). The gel and the buffer system was based upon that of Laemmli (1970) with the addition of 2mM EDTA to chelate metal ions which may interfere with the polymerisation of acrylamide and cause aggregation of proteins (Douglas and Butow, 1976). Polyacrylamide of molecular weight up to 5×10^6 Da was added to 0.5% (w/v) to increase the strength of the separating gel. Protein samples (20-100ul) were mixed with 5ul of 5% 2-mercaptoethanol in water, 5ul of 10% SDS, 5ul of 0.1% Bromophenol blue containing a drop of glycerol, and incubated in a boiling water bath for one minute. Samples were then applied to gels and electrophoresed at 100V for one hour followed by 50-70V overnight.

Gels were fixed in 20% methanol (v/v), 10% acetic acid (v/v) for 15 mins, stained at 55^{o}C in 55% methanol (v/v), 10% acetic acid (v/v), 0.25% Coomassie brilliant blue R (w/v) for fifteen minutes, then destained until the gel background was clear in 5% methanol (v/v), 7.5% acetic acid (v/v), also at 55^{o}C.

For determination of molecular weights, gels were calibrated with standard molecular weight marker proteins ranging from 14-200 KDa. The standard curve shown in fig. 2.3 of electrophoretic mobility on 12% SDS PAGE against the log of the molecular weight was obtained. This curve could then be used for molecular weight determination in the range 10-100 KDa.

2.2.4 Preparation of Fluorescent GM_1 Ganglioside

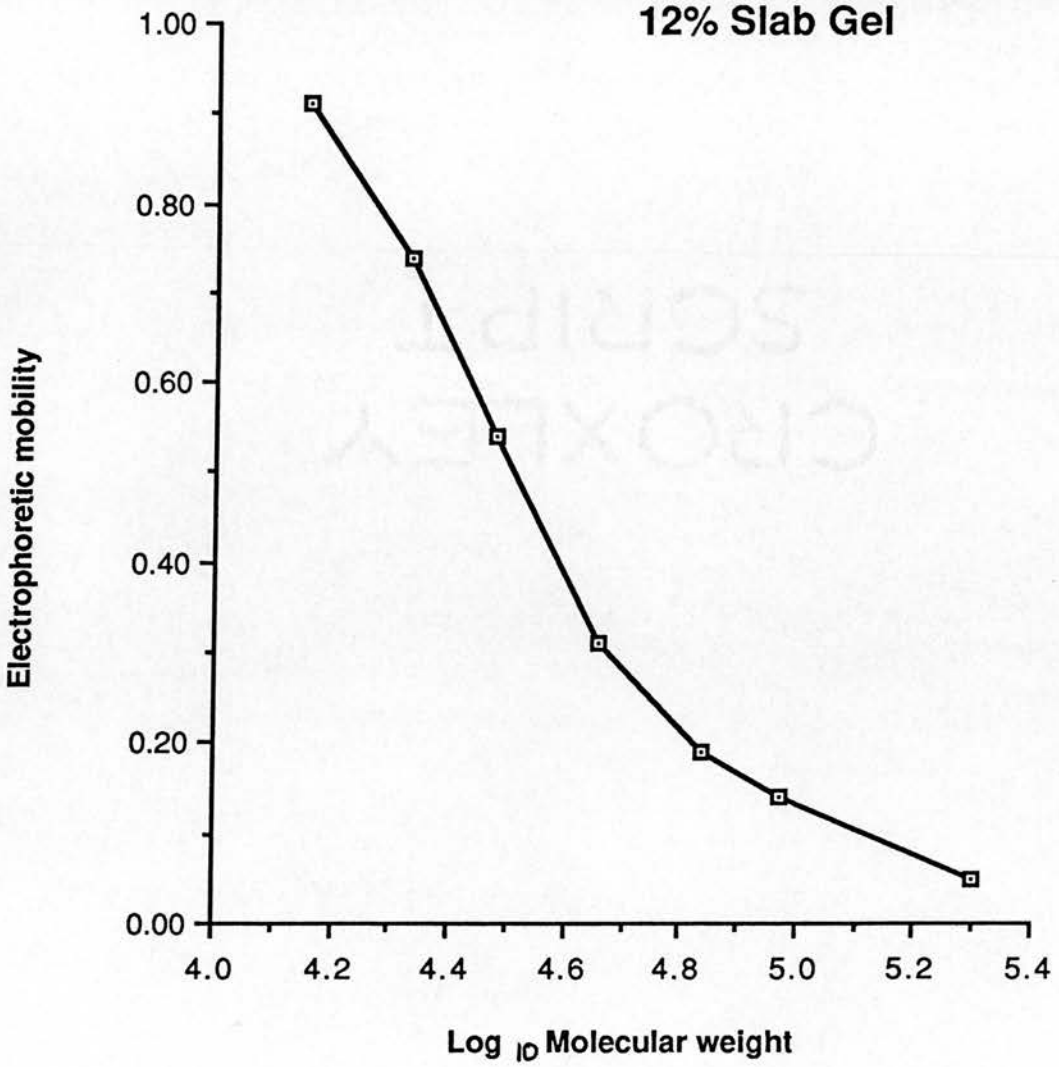
A fluorescent GM_1 ganglioside derivative was made according to the method of Spiegel (1985; 1987).

Freeze dried GM_1 ganglioside (3 mg) was dissolved in 3ml of 100mM sodium acetate buffer (pH 5.5) containing 150mM NaCl and 2mM $NaIO_4$ and left to react for 30 minutes at $0^{o}C$. This procedure produces oxidised GM_1. The reaction was stopped by adding 0.3ml of a 50% aqueous glycerol solution and the final mixture was dialysed overnight in standard dialysis tubing with three changes of three litres of water. Then the oxidised and dialysed GM_1 was lyophilised. The oxidised GM_1 was then dissolved in 3ml of PBS and fluorescein thiosemicarazide was added to a concentration of 5mM. The solution was then incubated overnight at $4^{o}C$ followed by dialysis as before this time with four changes of three litres of PBS. The material was then reduced with 10mM sodium cyanoborohydride for 15 mins at $23^{o}C$ followed by dialysis against three changes of three litres of distilled water and was then analysed by thin layer chromatography (T.L.C.).

2.2.4.1 Analysis of Fluorescent GM_1 Ganglioside

The fluorescent ganglioside formed a fluorescent 'precipitin line' with unlabelled cholera toxin using the Ouchterlony technique. Gangliosides are able to form micelles which might contain both labelled and unlabelled gangliosides. The binding might therefore represent only binding between toxin and unlabelled ganglioside. Therefore it was important to determine the proportion of unlabelled GM_1 molecules in the preparation.

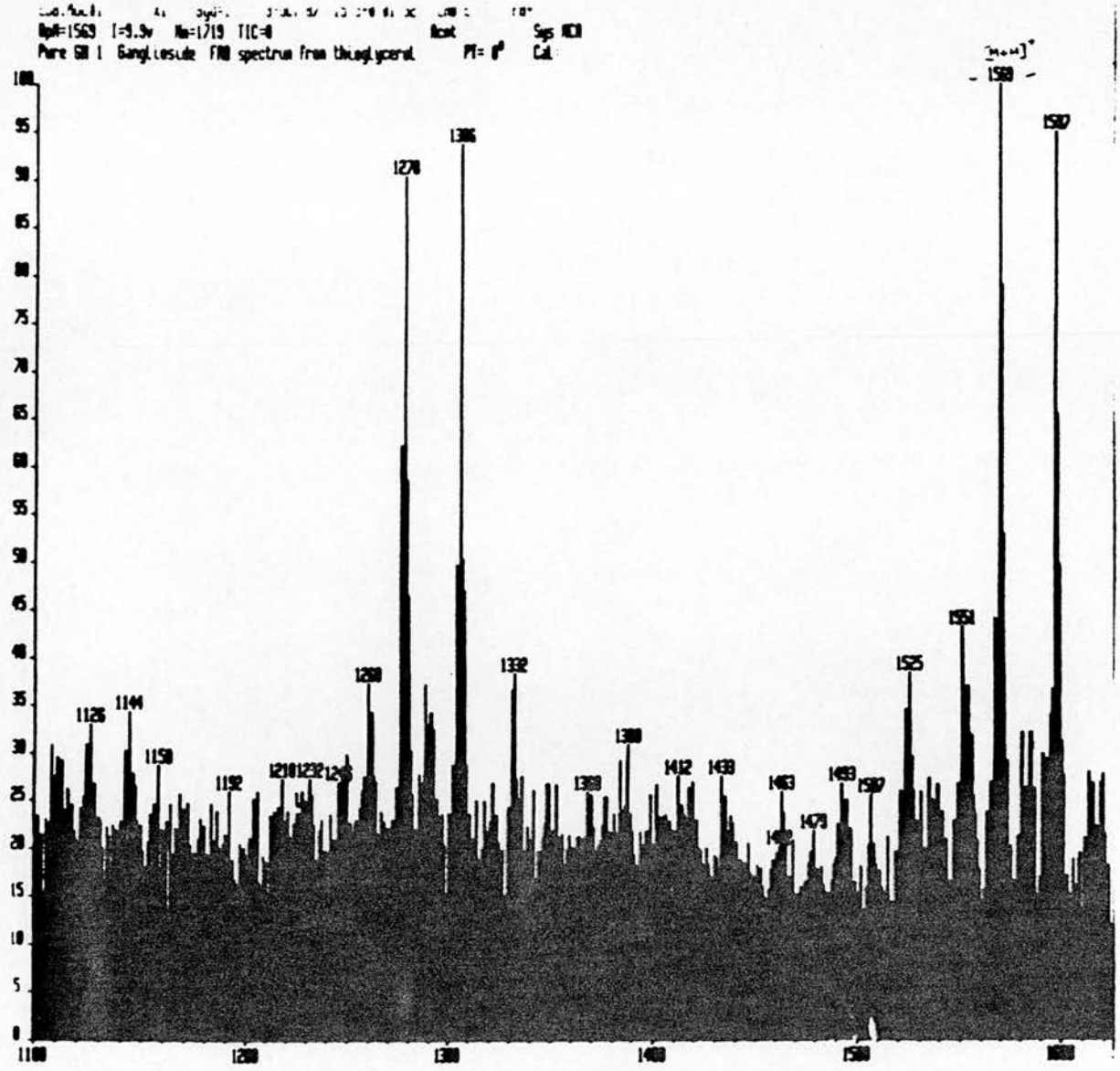
FIG 2.3 **Molecular Weight Calibration Curve**



The sialic acid determination assay of Svennerholm (1963) was used to determine unlabelled ganglioside present in the sample. However, this assay yielded no positive results, probably because the sialic acid residue had been modified by the labelling process and did not react with resorcinol in this assay. Another method used to determine the amount of unlabelled ganglioside present was thin layer chromatography (section 2.2.7) and a sensitive overlay technique with radioactive cholera toxin (section 2.2.8) but both derivatised and underivatised samples chromatographed with the same run front and could not be separated by T.L.C.

Samples of unlabelled GM_1 ganglioside and derivatised ganglioside were sent to the Mass Spectroscopy unit at Swansea run by the Science and Engineering Research Council. They used the technique of fast atom bombardment mass spectroscopy to determine the molecular weight ions. They obtained a trace for the unlabelled GM_1 ganglioside with a molecular weight of 1569Da which is the molecular weight of GM_1 plus a hydrogen ion (see Fig.2.4). The derivatised GM_1 failed to give any spectra under the conditions they used and they had insufficient material to try further solvents. Therefore, to date it has not been possible to characterise fully the derivatised GM_1 sample and determine the ratio of unlabelled GM_1 to labelled GM_1.

Figure 2.4 Fast Atom bombardment Mass Spectroscopy trace of GM_1 ganglioside indicating molecular ion of mass 1569 daltons, showing a pure preparation of the ganglioside



2.2.5 Preparation of IgG from Rabbit Serum

The polyclonal rabbit serum (van Heyningen, 1976) contained erythrocytes which obscured initial labelling experiments with colloidal gold, so an IgG fraction was prepared according to the method of Johnstone (1986).

Serum (50 ml) was warmed to 25°C and 9g of sodium sulphate was added to make an 18% w/v solution. The mixture was stirred gently and left for 30 min at 25°C. The solution was then centrifuged for 30 min at 3000g at room temperature, the supernatant discarded and the volume of the pellet made up to 25ml with distilled water. The solution was again warmed to 25°C and sodium sulphate was added to make a 14% w/v solution, the solution was left for another 30 min at 25°C. The centrifugation step was repeated and again the supernatant was discarded. The precipitate was redissolved in water and made up to a volume of 15ml and dialysed against three one-litre changes of 0.07M sodium phosphate buffer pH 6.3.

A 1.6cm x 30cm column was filled with DE52 Sepharose and equilibrated with the sodium phosphate buffer. The 15ml sample was added and 10ml fractions were collected and their A_{280} absorbance measured. The fractions containing protein were redialysed against 0.07M sodium phosphate pH 6.3 and lyophilised.

The lyophilised samples were dissolved in a total volume of 10ml, reapplied to the DE52 column in sodium phosphate buffer. Four fractions were eluted containing the protein. These were pooled.

2.2.6 Preparation of ^{125}I-labelled Cholera Toxin

Radioactive cholera toxin was made by the chloramine-T method (Greenwood et al., 1963).

Cholera toxin (20ul of 2mg/ml) was incubated on ice with 50ul of 16mg/ml chloramine-T solution and 50ul of iodide (0.5 mCi) in 100mM Tris-HCl 40mM NaCl pH 7.5 for 2 min. The reaction was stopped by addition of 50ul of 0.2M sodium iodide and 50ul 2.5mg/ml sodium metabisulphite.

The unconjugated iodide was separated from conjugated protein by passing 200ul aliquots through a 1ml column of Biogel P-6DG in an Eppendorf tube and centrifuging in an MSE bench centrifuge at 300g for 2 min at room temperature.

Then the radioactivity in a 1ul sample was measured in a gamma counter (LKB 1282). The stock 1mg/ml radioactive cholera toxin was stored at 4^{o} in a lead lined container. To check that both subunits were labelled, the toxin was run on a SDS PAGE 12% gel and stained to show the protein bands and dried down in a LKB slab gel dryer and exposed to Kodak X-ray film at -70^{o}C overnight. The autoradiograph was then developed and checked against the gel (see fig. 2.5).

2.2.7 Thin Layer Chromatography

Chromatographs of underivatised and derivatised GM_1 ganglioside (1-5ug) were routinely run on 10cm x 10cm glass backed silica gel 60 HPTLC plates in chloroform: methanol: 0.02% (aq) calcium chloride 60:40:10 to about 1cm from the top of the plate.

The plates were either exposed to iodine vapour which labelled any lipid or sprayed with the resorcinol reagent and covered and placed at 100^0C for 10 min which would specifically stain the sialic acid residues. Different solvents and proportions of solvents were also tried in order to attempt to resolve underivatised GM_1 from fluorescein labelled GM_1 (Ledeen and Yu, 1982). The two GM_1 samples always had the same mobility with all conditions tried (see section 2.2.4).

2.2.7.1 Overlay of T.L.C. plates with ^{125}I-labelled Cholera Toxin

After chromatographs of ganglioside GM_1 samples were run as described in 2.2.7. The glass plates were soaked for 1-2 hr in a 1% BSA, 0.2% Tween solution to block all non specific binding sites. Then the plates were incubated with 50 mM Tris-HCl buffer (with 150mM NaCl and 1% BSA) containing the radioactive cholera toxin so that the initial radiaoactive measurement of the solution was 10^6 counts per minute per millilitre of buffer and this was left in contact with the plates for 30 min. The plates were then thoroughly washed in three changes of the Tris-HCl buffer (1 litre) before being dried and autoradiographed.

2.2.8 Indirect Immunofluorescent Detection of Cholera Toxin Bound to NIH 3T3 Cells

NIH 3T3 cells were grown normally and seeded out onto glass coverslips at a low density of 1 x 10^6 cells/ml of medium, one day prior to use. The cells were then fixed in 3% paraformaldehyde in PBS for fifteen minutes at room temperature. Different coverslips were then incubated with 100ul of a range of cholera toxin dilutions (10 ug/ml - 30 ug/ml) for 30 min at room temperature. The coverslips were then washed in 3 changes of PBS.

FIG 2.5

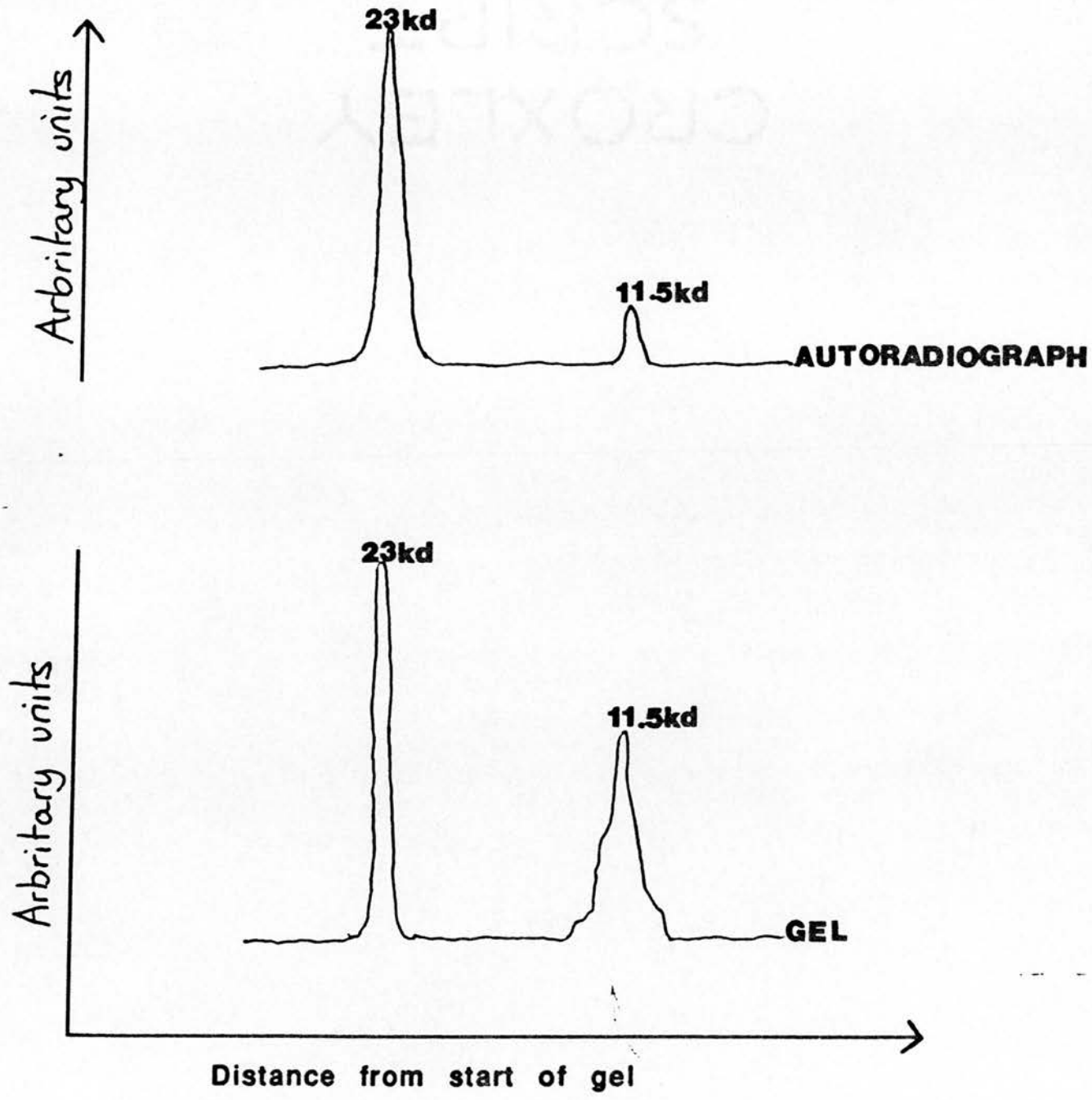


Fig.2.5 Densitometric traces measured on a Joyce Loebl Chromoscan of a 12% gel containing ^{125}-I-labelled cholera toxin and the corresponding autoradiograph showing that both A and B subunits are labelled.

An incubation with rabbit anti-(cholera toxin) antiserum followed, dilution being either 1/10 or 1/50 in PBS, for 30 min at room temperature. This was followed by extensive washing and a final incubation of the coverslips with FITC-labelled goat anti-(rabbit IgG) antiserum (1/30 dilution in PBS) for 30 min at room temperature.

The coverslips were then washed again and mounted onto slides and viewed with a Leitz fluorescence microscope (see fig. 2.6).

The cells were also labelled directly with the fluorescent (FITC labelled) cholera toxin (see section 2.2.2) at a dilution of 1/100 in PBS. Labelling of the cells with fluorescent toxin could be prevented by prior labelling with an excess of unlabelled toxin, demonstrating that a limited number of binding sites were present per cell.

2.2.9 Titration of NIH 3T3 Cells with Fluorescent Cholera Toxin

NIH 3T3 cells were grown normally and seeded out onto glass coverslips at a concentration of 60 000 cells/ml.

The fluorescent cholera toxin was serially diluted from (31.25 ug/ml up to 1 mg/ml) in PBS buffer. The cells were incubated with a 100ul aliquot of the toxin for fifteen minutes at room temperature and then washed several times in PBS. The coverslips were then mounted onto slides.
The slides were observed by using a Vickers microscope connected to the laser in the Fluorescence Recovery After Photobleaching (FRAP) set up (see chapter 3, Fig.3.2). The collimating lens in the laser beam was set to give a wide illumination of the sample through the microscope objective and the light intensity from cells and background was measured by the photomultiplier tube and the voltage reading from this was recorded.

Fig.2.6 NIH 3T3 cells labelled with 30ug/ml cholera toxin followed by a 1/10 dilution of anti-(Cholera toxin) antiserum in PBS and a 1/30 dilution of fluorescein-labelled anti-(rabbit IgG) antiserum in PBS.

Bar = 0.1um

At least ten individual measurements for each cholera toxin concentration were recorded and the mean and standard deviation for each was calculated.

The mean voltage values relating to the amount of fluorescence were plotted against the cholera toxin concentration (see Fig.2.7).

2.2.10 Protein Assay

The Bradford (1976) protein assay was routinely used. A stock solution of Bradford reagent was made. This contained 100mg Coomassie blue in 50ml of 95% ethanol and 100ml of phosphoric acid. This was kept at $4^{o}C$.

The working solution involved diluting 18ml of the stock reagent up to 100ml with distilled water. To remove aggregates of Coomassie dye, this solution was filtered through Whatman No. 1 paper before use. Using the working solution, a standard curve was made with standard BSA solutions (see fig. 2.8). The assay involved 500ul of test sample and 2.5ml of working reagent rapidly mixed. The amount of protein in a solution was related to the absorbance at 595nm read in a Cecil spectrophotometer after the mixed solution had stood for 30 minutes and was read against a reagent blank.

2.2.11 Ouchterlony Technique

In order to test that the fluorescent derivatives of cholera toxin and ganglioside GM_1 bound to underivatised GM_1 and cholera toxin respectively, the double-diffusion technique was used (Ouchterlony, 1958). Binding was indicated by the formation of a 'precipitin' line between the two solutions of interest.

Stock 3% agar was made up in distilled water containing 0.02% sodium azide and kept in 5ml solidified aliquots. When required, 5ml of 0.1M sodium phosphate buffer (containing 1.8% sodium chloride) pH 7 was added to a 5ml agar aliquot.

The mixture was heated in a boiling water bath until the agar had dissolved. The solution was then allowed to cool to about 60^{o}C and 2ml of diluted agar was applied per acid washed slide placed on a black 'level' table. Slides were then placed in a humid box to set. Holes were cut with an Ouchterlony cutter. Up to 6ul of a cholera toxin/ganglioside solution was applied to the wells. The slides were then returned to the humid box overnight after which they were examined for any signs of binding between the two solutions being tested.

2.2.12 Blotting of Proteins onto Nitrocellulose (Western Blotting)

Gels were electrophoresed as described in section 2.2.3 Prior to blotting, a nitrocellulose sheet 20cm x 20cm (Anderson and Co. Ltd.) together with two 'Scotch-Brite' pads were soaked in the transfer buffer (0.02M Na_2HPO_4; 0.02% SDS (w/v); 20% MeOH (v/v) with some thick filter paper sheets for 2 hours. When the gel had electrophoresed, it was sandwiched against the nitrocellulose between the filter paper sheets and the two pads with a clamp. This cassette was then placed in the LKB blotter with the nitrocellulose nearer to the anode. Transfer of proteins was achieved after 2-3 hr with a 10% voltage giving an approximate current of 0.6 Amps-1.5 Amps.

After the time allotted, the cassette was removed and the nitro-cellulose either stained to locate the protein bands or placed in blocking buffer prior to immunostaining (see chapter 5, table 5.0).

FIG 2.7

Concentration Dependence of Binding of Fluorescent toxin against NIH 3T3 cells

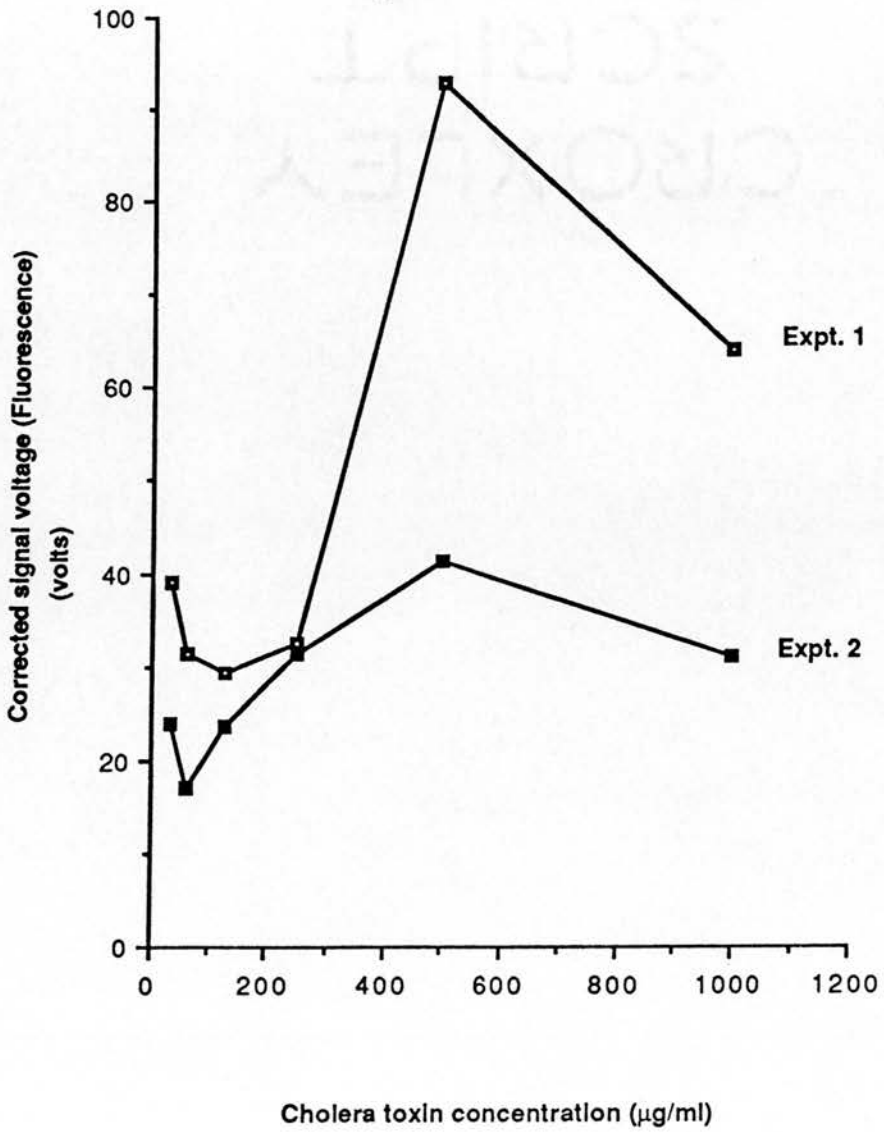
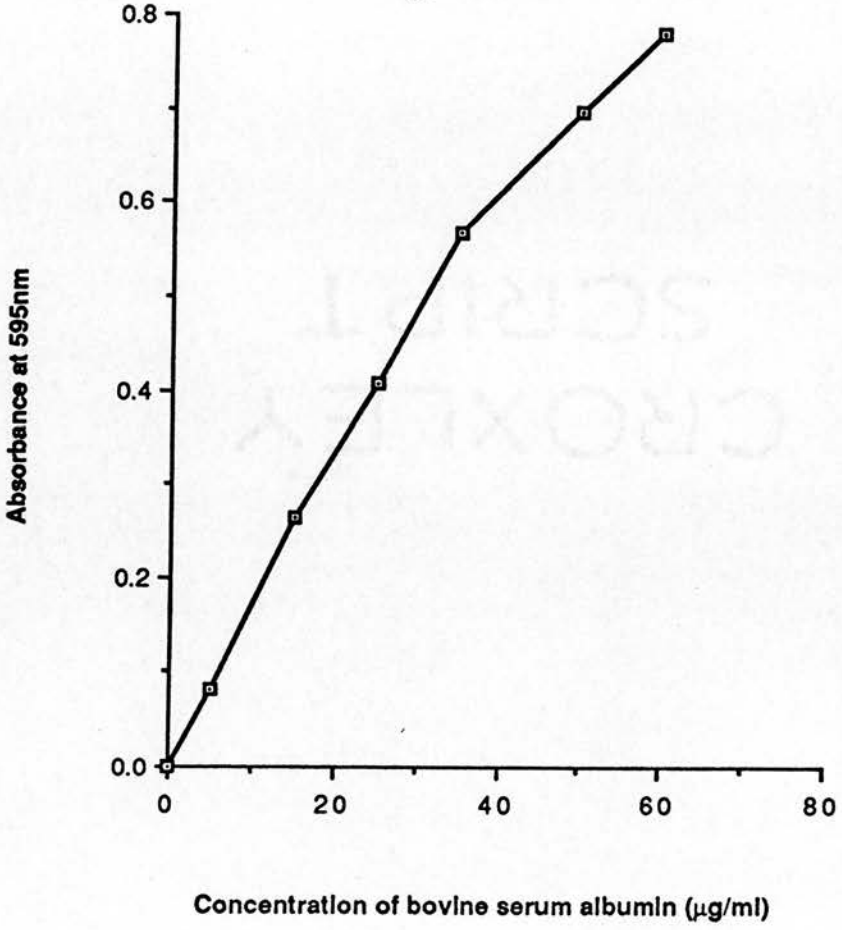


FIG 2.8 Standard Curve for Protein assay by Bradford Method



The staining solution consisted of 2g Amido Black 10B (LKB) in 200ml of a solution of methanol/acetic acid/distilled water 9:2:9; v/v/v. Staining occurred within ten minutes. Destaining of the background was carried out in methanol/acetic acid/distilled water; 9:2:9; v/v/v, until clear bands on a light background were observed (Towbin et al., 1979).

2.2.13 Preparation of Fluorescent BSA in 90% Glycerol

BSA (1mg) was dissolved in 5ml of 0.25M phosphate buffer pH 8.0 then 5.8mg of fluorescein isothiocyanate (FITC) was added and the mixture was incubated overnight at 4^{o}C.

Pharmacia G-25 columns were equilibrated with PBS and the incubated mixture was passed through to separate conjugated protein from the individual components. The FITC-labelled BSA came through in the void volume.

Finally a known weight of the FITC-BSA solution was mixed with a known weight of glycerol 90% weight/weight. This mixture was used to calibrate the laser in the FRAP experiment described in Chapter 3 (Section 3.5.1).

CHAPTER 3

USE OF FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING TO MEASURE THE LATERAL DIFFUSION OF CHOLERA TOXIN AND GM_1 GANGLIOSIDE ON NIH 3T3 CELLS

CHAPTER 3

USE OF FLOURESCENCE RECOVERY AFTER PHOTOBLEACHING TO MEASURE THE LATERAL DIFFUSION OF CHOLERA TOXIN AND GM_1 GANGLIOSIDE ON NIH 3T3 CELLS.

3.1.0 Introduction

This chapter describes the measurement of the lateral mobility of inserted fluorescent GM_1 ganglioside into the cell membrane of fibroblasts. The first measurement of the lateral mobility of fluorescent cholera toxin bound to fibroblasts, is also described. The lateral mobility measurements were carried out using the technique of Fluorescent Recovery after Photobleaching (FRAP).

The information that can be gained from lateral mobility measurements is described, showing results obtained by previous workers who examined fluorescent ganglioside in different systems (cellular and non cellular).

A brief historical review follows on the theoretical development of the existence of lateral mobility on cell surfaces.

The FRAP technique is described indicating the important measured variables which allow calculation of the lateral mobility of fluorescent cell surface molecules as well as a description of the first experimental measurement of lateral mobility.

The FRAP equipment is then described followed by the results of the measurements I made and a discussion of these results.

3.1.1 Why Measure the Lateral Mobility of Cholera Toxin GM_1 Ganglioside Complexes?

The intermediate steps between cholera toxin binding to its specific ganglioside receptor and the ensuing activation of adenylate cyclase, remain unclear.

The cellular interactions of the ganglioside receptor within the membrane (its possible attachment to transmembrane components) and how cholera toxin might alter the receptor to bring about the final effect are unknown. Therefore the measurement of mobility of *in situ* cell ganglioside GM_1 by use of the fluorescein-labelled cholera toxin in comparison with that of inserted fluoresceinated GM_1 ganglioside may provide an opportunity to quantitatively examine the receptor and the toxin receptor complex in cells. Any differences might give clues as to (1) How the cholera toxin altered receptor conformation and (2) How the cholera toxin-GM_1 complex may interact with other molecules.
These experiments do assume that inserted ganglioside is in the same configuration as *in situ* ganglioside. Inserted ganglioside GM_1 has been widely used in cells lacking the ganglioside to allow binding of cholera toxin to occur with ensuing activation of adenylate cyclase, (Fishman, 1982).

3.1.2 Previous work on GM_1 Mobility

The mobility of ganglioside GM_1 was originally hypothesised after observations of patching (punctate appearance of receptor once ligand is bound) and capping (grouping of receptors to one pole of the cell) following cholera toxin binding in lymphocytes. Table 3.0 summarises these observations:

Table 3.0 Table Summarising the Cholera Toxin/Ganglioside Patching and Capping Observations made in Lymphocytes

CELLS	EXPERIMENTAL SYSTEM/OBSERVATIONS	REF.
Human/Murine lymphocytes	Three-layer immunofluorescence procedure with visualisation using fluorescent rabbit anti-horse serum. The amount of capping was reduced for a particular cholera toxin conc. if the number of GM_1 molecules was increased. Cytochalasin B inhibited capping.	Revesz and Greaves, 1975
Rat lymph node cells	Fluorescein-labelled cholera toxin. Inhibition of redistribution by several metabolic, microtubule and microfilament inhibitors.	Craig and Cuatre-casas, 1975
Human lymphocytes	Inserted fluorescent-labelled synthetic glycolipid (DANSyl-gangliosidoide). Redistribution is sensitive to colchicine. Toxin receptor complex caps together with anti-immunoglobulin receptors.	Sedlacek et al., 1976
Mouse lymphocytes	Three-layer immunofluorescence procedure with visualisation using fluorescent swine anti horse serum. Capping is inhibited by azide, low temperature and cytochalasin B. Co-capping with a-actinin observed.	Kellie et al., 1983
Mouse thymocytes	Inserted fluorescent ganglioside GM_1. Co-capping of exogenous GM_3 ganglioside observed.	Spiegel et al., 1984

In summary, the experimental evidence indicates that capping of the cholera toxin receptor complex is an energy dependent process with involement of the microfilament system, possibly actin. The possible interaction of GM_1 with the microfilament system must be indirect as the hydrophobic region of the glycolipid is not thought to span the bileaflet membrane. These experiments also indicate that gangliosides may self-associate in the membrane forming aggregates with themselves or with other receptors but the physiological explanation for this is not clear.

Previous workers have examined the lateral mobility of inserted ganglioside and their values for the diffusion coefficient obtained are summarised in Table 3.1. These measurements were made in order to look at properties of the plasma membrane. The results show that inserted fluorescently labelled GM_1 ganglioside has a similar lateral diffusion coefficient to other membrane inserted lipid probes such as diI (3.3'-diacylindocarbocyanine iodide) which is similar to a diacyl phospholipid but the headgroup is more bulky as it contains the chromophore.

Eldridge et al., (1980) compared the plasma membrane of normal 3T3 fibroblasts with those which had been transformed by SV40 virus. One method of analysis was to compare the lateral diffusion of a fluorescein GM_1 derivative inserted into the plasma membrane of both types of cell. He found no difference between the mobility of the ganglioside in normal and in transformed cells. This experiment could imply that there are no major cell surface differences or it might indicate that ganglioside inserts into a similar region in both cell types.

Schlessinger et al., (1977b) measured the lateral mobility of a fluorescein ganglioside analogue inserted into chicken embryo fibroblasts in areas rich or poor in an immobile cell surface glycoprotein to see whether there was any interaction of the glycolipid with the glycoprotein. In this system the amount of glycoprotein did not affect the ganglioside mobility. In this experiment too, ganglioside may insert itself in distinct regions of the cell surface which are unaffected by the level of glycoprotein.

Spiegel et al., (1984), measured the lateral mobility of a fluorescent GM_1 derivative inserted into the cell membrane of a primary culture of human fibroblasts to show that it was indeed stably inserted (criterion was to obtain accepted mobility of $10^{-8}cm^2s^{-1}$ without any immobile species). She showed that GM_1 mobility depended upon the density of the fibroblasts. When there was a comparatively high density of fibroblasts the ganglioside was immobile.

Finally, Goins et al., 1986, examined the mobility of a fluorescent GM_1 ganglioside inserted into artificial lipid membranes (Dimyristoylphosphatidylcholine (DMPC) vesicles) and examined whether cholera toxin binding had any effect on the lipid mobility in this system. He found that cholera toxin binding did not significantly reduce ganglioside mobility. This experiment may indicate that no major conformational change occurs in the toxin or that there is no restriction to diffusion ie no cytoskeletal interactions to bound ganglioside in an artificial cell membrane system which effect ganglioside mobility within that membrane.

3.1.3 The Present Study

As mouse fibroblasts are easy cells to grow, were already established at Unilever, and are the cells which have been used most for lateral mobility measurements, I decided to use them as my model cell.

I re-examined the mobility of ganglioside GM_1 by inserting the fluorescent ganglioside into the cell but I was also able to examine the mobility of endogenous cell-localised GM_1 using fluorescent cholera toxin, thus also looking at the toxin-receptor complex.
I was able to determine to some extent whether *in situ* gangliosides and inserted ganglioside behaved in the same way, and whether toxin binding to ganglioside in cell membranes rather than liposomes had an effect on mobility.

Table 3.I The Mobility of Fluorescent Lipid probes GM_1 and diI in Different Cell Systems

PROBE	CELL PREPARATION	MEAN DIFFUSION COEFFICIENT ($X\ 10^{-9} Cm^2 S^{-1}$)	% MOBILITY	REF.
$FL\text{-}GM_1$	Human fibroblasts	10	100	Spiegel et al. 1984
$FL\text{-}GM_1$	Mouse fibroblasts			
$FL\text{-}GM_1$	Normal	4.97	92	
	Transformed	4.24	89	Eldridge et al. 1980
$FL\text{-}GM_1$	Chicken embryo fibrobast			
	+immobile glycoprotein	0.42		
	-immobile glycoprotein	0.46	NOT QUOTED	Schlessinger et al. 1977b
$FL\text{-}GM_1$	DMPC vesicles	4.7	>80	Goins et al. 1986
$diI\text{-}C_{18}$	L-6 rat peritoneal mast cell	8	NOT QUOTED	Schlessinger et al. 1976
$diI\text{-}C_{18}$	Mouse 3T3 cell line	6	86%	Wolf et al.1980

NB All values quoted are the mean experimental values obtained. The standard error of measurement varied from 15% to 30%. Measurements were carried out at room temperature ($20\text{-}22^0 C$).

3.1.4 Brief Review of the Evidence for Existence of Lateral Mobility of Components on the Cell Surface.

The first definitive experimental demonstration that proteins were able to diffuse laterally in the cell membrane came from the antigen diffusion experiment performed by Frye and Edidin (1970). In this well known experiment, mouse and human cells were fused with Sendai virus to form a heterokaryon bearing both mouse and human surface antigens, which were then examined by the indirect fluorescent antibody method. By forty minutes after fusion, total mixing of both parental antigens had occurred in over 90% of the heterokaryons. One criticism of this experiment could be that the virus induced fusion had perturbed the normal topology of the membrane.

Singer and Nicolson (1972) postulated the fluid mosaic model of cell membranes. This model required that the membrane was not rigid but in fact a dynamic structure consisting of protein and lipid in non covalent associations with one another. Membrane components must be mobile to serve signal transduction and distribution and redistribution of matter within the cell and between the cell and its environment. However, there are specialised areas on plasma membranes such as junctions or pits which might indicate immobilised membrane components or rigid cell areas or both. A most striking example of a cell where such specialised areas exist is an intestinal epithelial cell.

Another phenomenon on the cell surface which indicates that there is mobility of components is that of patching and capping first observed by Moller in 1961 (Moller, 1961). It was not till 1971 that capping was seen as an indication of lateral mobility of cell surface immunoglobulin (Taylor et al., 1971). One theory of how capping could occur is postulated by Bretscher who stated that lipids migrate to the poles of the cell where they are internalised and in doing so some protein components are also caught up in the flow; (Bretscher, 1976). However, Bretscher predicts that proteins moving as slow as $10^{-10}cm^2s^{-1}$ would cap without antibody since they could not overcome the flow of membrane lipids, but lateral diffusions of proteins less than $10^{-10}cm^2s^{-1}$ have been reported for uniformly distributed proteins (Edidin, 1981). Patching is independent of cell metabolism but generally requires a divalent antibody but the collection of patches into a cap is metabolically driven and much evidence exists to show that it depends upon the cytoskeleton (Bourguignon and Singer, 1977, Edidin, 1981). Capping has been observed for cholera toxin receptors only in lymphocytes (see table 3.0).

Receptors for many regulatory agents, eg. peptide hormones, neuro-transmitters and antigens have a dual function of recognising the appropriate signal and initiating the biological response to its presence. Originally, it was thought that the receptor and the catalytic domains of the effector molecules formed a macromolecular complex. However, certain experimental data do not fit with the hypothesis of the existence of this complex, especially data concerning those receptors which recognise hormones activating adenylate cyclase. When two hormones which both activate adenylate cyclase in the same cell, but bind at different receptors, are added to that cell together, the maximum level of cAMP produced is the same as if only one of the hormones had been used.

This result indicates that the pool of effector molecules is not the same as that of receptor molecules and that the receptor may migrate once bound with hormone to the effector, the adenylate cyclase enzyme. Therefore, the amount of cAMP which may be produced is limited by the number of catalytic subunits of adenylate cyclase. This idea was postulated by Jacobs and Cuatrecasas (1977) and is known as 'The Mobile Receptor Hypothesis'.

This theory eliminates the requirement for single receptor-effector complexes and allows for the explanation of how a single enzyme e.g. adenylate cyclase may be regulated by several different receptors and also the converse idea: how a single receptor can regulate several different and apparently independent membrane functions. This theory has been recently discussed by Peters (1988) who confirms that such a model is still possible despite small measured diffusion coefficients of the proposed mobile receptor eg b-adrenergic receptor on red blood cell. Peters presents evidence for cellular subdomains on the cell surface in the erythrocyte membrane and evidence of the existence of a laterally mobile signal carrier which could move from the receptor to the effector molecules within these small subdomains.

Lateral mobility on the cell surface is an accepted phenomenon and has now been widely studied.

3.2.0 The Fluorescence Recovery After Photobleaching Technique.

Fluorescence microphotolysis, the technique devised by Peters in 1974 (Peters et al., 1974) to measure lateral diffusion of cell surface molecules on erythrocyte ghosts,

is also known as Fluorescence Photobleaching Recovery (FPR) or Fluorescence Recovery after Photobleaching (FRAP). The latter term will be used in this description.

The principle of the FRAP technique is simple. Molecules of interest on the cell's surface are fluorescently labelled and the fluorescence from a very small region on the surface (usually a circular area 2um in diameter) is excited by a laser beam which is focused on the small spot through the objective of a microscope.
Fluorescence is detected with a photomultiplier tube positioned beyond the image plane of the objective. A pinhole apperture in the image plane is confocal with the laser spot and reduces stray light and out of phase fluorescence. To avoid photobleaching at this stage the laser is attenuated to a few microwatts. Once a steady reading of fluorescence is achieved a fraction of the fluorophores is irreversibly photochemically bleached by increasing the laser power incident on the membrane by about 1000 fold for 50-200 milliseconds, after which the laser power is returned to its original low intensity.

The rate of recovery of fluorescence which results from transport of unbleached fluorophores into the bleached area from the surrounding area is measured and from this the diffusion or flow coefficients can be determined (see fig. 3.1 for a diagrammatic representation of the bleaching and recovery process and data analysis section for mathematical interpretation). The fluorophore used is usually fluorescein or rhodamine and the wavelength for excitation of the fluorophore is chosen such that it is not absorbed by any components of the cell.

3.2.1 Theory of FRAP and Data Analysis.

In 1976, Axelrod et al. provided the theoretical basis and simple analysis of the results derived from FRAP experiments. From analysis of the kinetics, one can differentiate between flow and diffusion, determine the immobile fraction and measure diffusion coefficients over a wide range (10^{-6} to $10^{-12} cm^2 s^{-1}$) over distances of a few microns.

For two dimensional diffusion Einstein states that the mean square distance x^2 moved in a short time, t, is given by:

$$x^2 = 4Dt$$

or

$$D = x^2/4t$$

where D is the lateral diffusion coefficient.This is similar to the equation used to determine the lateral diffusion coefficient from FRAP experiments derived by Axelrod et al, (1976):

$$D_L = w^2 \, y/4T_{1/2}$$

Where

D_L = lateral diffusion coefficient

w = radius of the bleaching area at $1/e^2$ (0.135) intensity and the beam has a Guassian profile

$T_{1/2}$ = time taken for fluorescence to reach its half maximal value

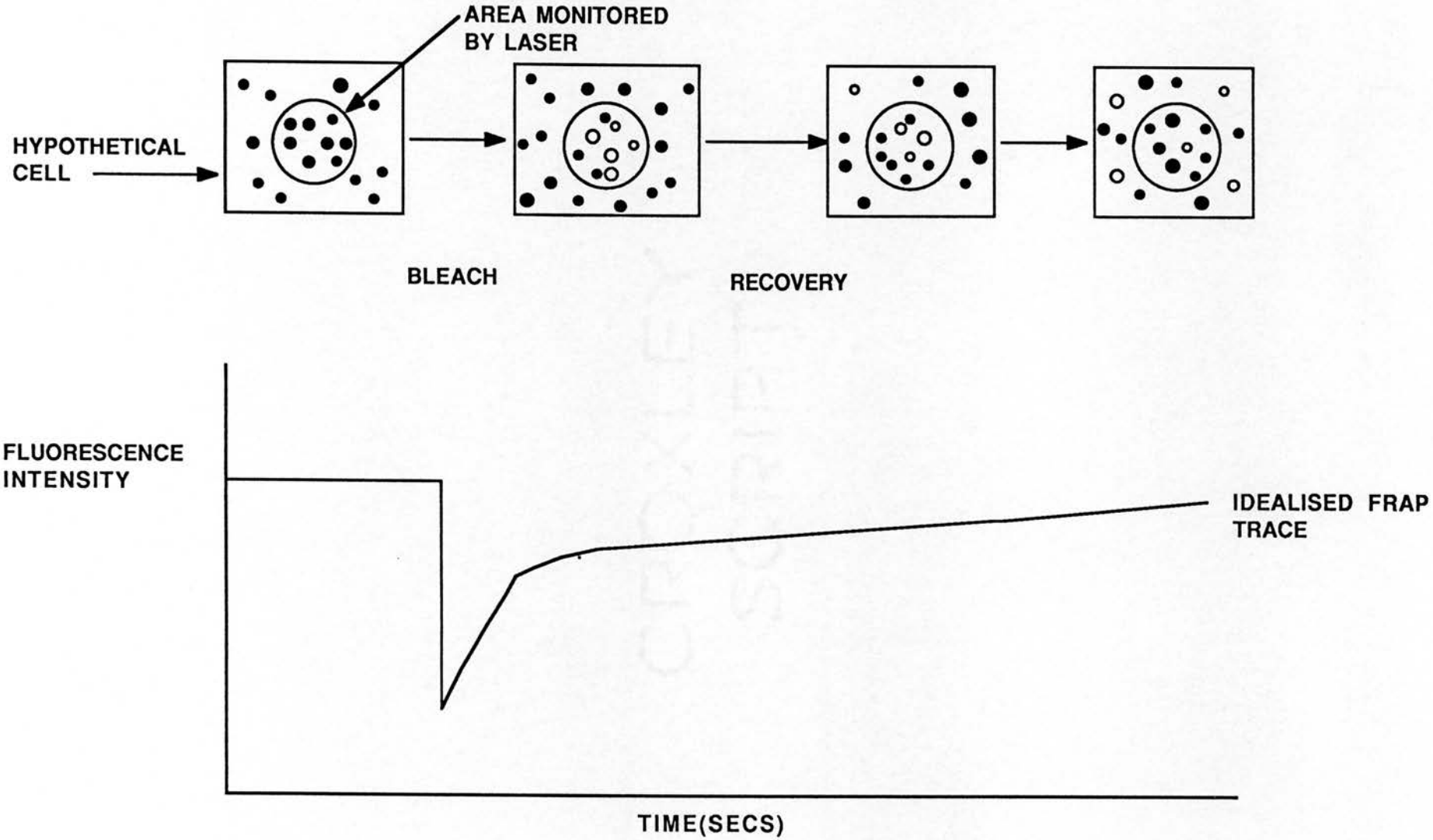


FIG 3.0 PRINCIPLE OF FRAP

y = a constant that depends on the beam profile and the bleaching parameter K, which in turn depends on the % bleach (which depends on laser intensity, duration of bleaching pulse, fluorescent extinction coefficient and the quantum efficiency of bleaching)

The following assumptions were made;

1. Photobleaching is a simple, irreversible first order reaction.

2. The amount of fluorescence bleached during the recovery period is less than 1% of the total fluorescence in the spot.

3. The bleaching time is less than 10% of the characteristic diffusion time.

4. There is no flow occurring.

The fraction of mobile molecules is given by:

$$R = \frac{F(\infty) - F(o)}{F(-) - F(o)}$$

Where $F(-)$ = fluorescence intensity before bleach

$F(o)$ = fluorescence intensity immediately after the bleach

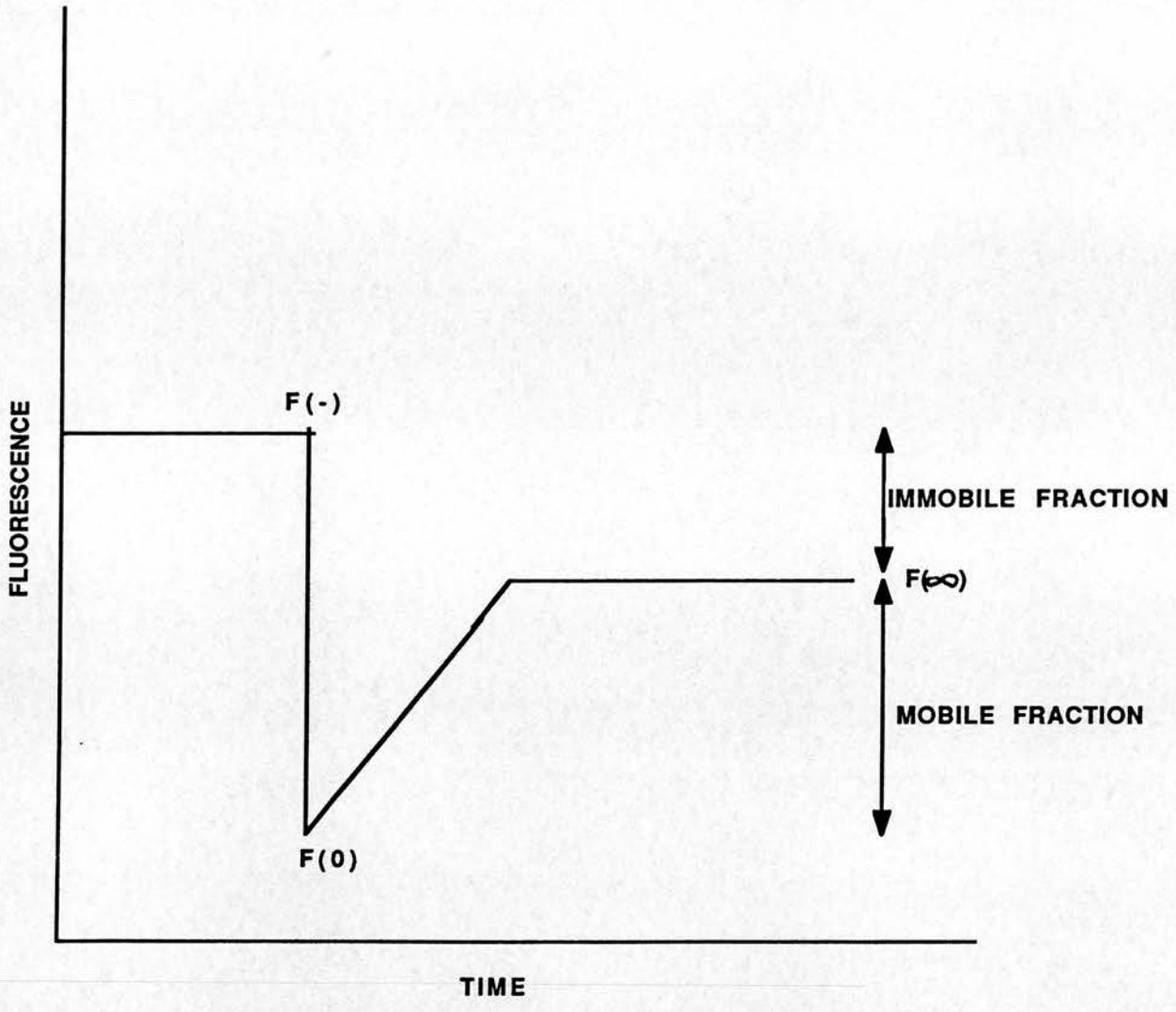
$F(\infty)$ = fluorescence after recovery

R = percentage mobile molecules (percentage recovery)

(see fig. 3.1)

In 1982, Yguerabide et al. (1982), developed a linear least squares analysis for even more precise analysis of FRAP data, which was programmed into the computer for analysis of the traces. Essentially, the Yguerabide analysis fits the experimental data to a theoretical straight line estimated from the data and an iterative process occurs until the best fit is obtained between the experimental and the theoretical data. The residuals presented with the fitted trace give an idea of the data fit to the straight line. As can be seen from fig. 3.4 the traces obtained are quite noisy i.e. not smooth lines, but the program fits a line that approximates well to the data points.

Further information presented on the two traces from the computer analysis (Fig.3.4) are the percentage recovery i.e. the percentage of mobile molecules, the $T_{1/2}$ value and the % bleach, from which, using a plot of the variation of the % bleach and y, one can calculate the diffusion coefficient using the Axelrod equation.



IG 3.1 FLUORESCENCE RECOVERY CURVE INDICATING THE IMPORTANT VARIABLES

3.2.1.1 Are FRAP Results Radiation-Induced Artefacts?

FRAP has been criticised as a technique to measure cellular diffusion on cell surfaces as there has been concern regarding possible radiation damage to the cell and temperature heating effects which it is argued may alter the diffusion values obtained.

A fundamental calculation by Axelrod (1977) clearly showed that the local temperature rise in cells is smaller than $0.03^{o}C$, even in the worst case where all absorbed light energy is converted to heat, so local heating within the cell is unlikely to be significant.
A number of indirect experiments have been carried out which clearly indicate that FRAP does not produce artefactual results because of cell damage. The area of membrane where the laser was focused was examined by scanning electron microscopy and no damage was found. Furthermore cell impermeability to trypan blue was also checked after FRAP measurements, also indicating no cell damage had occurred (Jacobson et al, 1978). Peters and Richter (1981), looked at the effect of FRAP on the fertilisation and early development of sea urchin eggs and could find no evidence of radiation damage. In some cases diffusion has been measured in the same cell by both FRAP and another method and the diffusion coefficient has been the same. Schlessinger et al. (1977a) measured the diffusion of a lipid probe in myoblasts by both FRAP and fluorescence correlation spectroscopy and found no difference in the results.

Therefore, no evidence at present is available to indicate that the FRAP procedure produces a significant perturbation of cell structure; low diffusion coefficient values obtained in cells is probably an indication of a protein or lipid constrained by interactions with other proteins or lipids within the membrane or on either side of the membrane (Edidin, 1987).

3.3.0 Initial Measurements of Lateral Diffusion of Cell Membrane Components.

In 1974, two independent groups simultaneously provided the first attempts at achieving quantitative data for lateral diffusion. Poo and Cone (1974), measured the diffusion of rhodopsin in rod photo-receptors, whereas Peters et al. (1974), devised a technique known as 'fluorescence microphotolysis' to try to measure the mobility of human erythrocyte membrane components in red blood cell ghosts labelled with the fluorescein isothiocyanite.

Poo and Cone (1974) found the diffusion coefficient of rhodopsin to be 3.5 x $10^{-9}cm^2s^{-1}$ at 20^0C, and they concluded that the membrane of frog outer segments was highly fluid.
They exploited the intrinsic properties of the rhodopsin chromophore, which was dark adapted and once bleached could not be regenerated. They measured the optical absorbance of both sides of a rod by using an alternator to shift the measuring beam from one side to the other. At the start of the experiment the absorbance on each side of the rod was essentially identical.

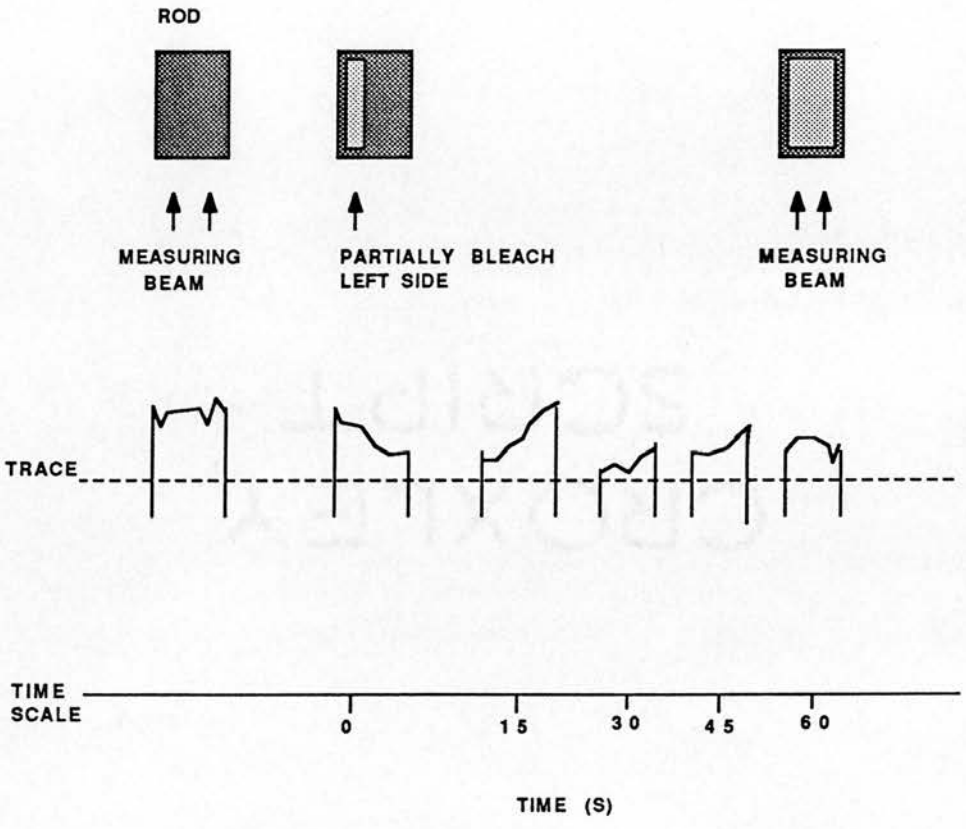
After increasing the measuring beam momentarily on one side and then monitoring absorbance as before, they noticed a decrease in absorbance of molecules on the side of the rod where they had increased the measuring beam momentarily. With time, the absorbance readjusted, increasing on the reduced side and decreasing on the unaltered side of the rod (see fig.3.2). As glutaraldehyde fixation prevented the readjustment in absorbance, this experiment clearly indicated that the rhodopsin molecules were diffusing from one side of the membranes to the other along the discs.

Poo and Cone also found that the diffusion did not occur lengthwise down the rod between discs. The diffusion coefficient was calculated using the distance from one side of the rod to the other and the time taken for equilibration in absorbance.

The fluorescence microphotolysis method of Peters et al., (1974) which also measured diffusion did not depend on the membrane having an intrinsic chromophore. In this experiment, the molecules of interest (the proteins of the erythrocyte ghost membrane), were fluorescently labelled then only half a ghost membrane was bleached and measurements were taken to see if unbleached material would diffuse into the bleached side of the ghost membrane. It was found that after 20 minutes of measuring, the labelled molecules appeared immobile within the timescale of the experiment and from the diffusion equation for a spherical surface Peters derived a diffusion coefficient of less than or equal to $2.3 \times 10^{-11} cm^2 s^{-1}$ at $23^{o}C$.

FIG 3.2

Schematic illustration of the results of the Poo and Cone experiment to measure rhodopsin lateral diffusion in mudpuppy rods.



Trace indicates redistribution of the bleached and unbleached chromophore to produce a lower overall signal.

Thus, the mobility of proteins on the erythrocyte membrane were found to be at least 1000 x slower than rhodopsin in frog rod outer segments. The differences in the two results may be due to;

1) The different lipid compositions of the two membranes (and therefore different viscosity). Erythrocytes contain more cholesterol and saturated fatty acids than most cells.

2) The presence of a cytoskeleton in the erythrocyte (the meshwork of proteins under the membrane may bind with molecules within the membrane).

3) The adverse effect on the cells of fluorescent probe addition.

Measurements of lateral diffusion of many proteins and lipids on a variety of cells and model systems have now been made and have been extensively reviewed by Peters (1981). These lateral diffusion measurements are similar to within an order of magnitude whether they have mean measured by FRAP or other techniques used to measure lateral diffusion such as fusion or electrophoresis (Cherry, 1979; Peters, 1981).

THE FRAP METHOD APPARATUS USED

3.4.0 Instrumentation

A diagrammatic representation of the apparatus used to measure lateral mobility is shown in fig. 3.3

The Laser (Model 85-1, Lexel Corp., Palo Alto, Calif. 94303)

The laser used was a 1 watt (all lines) continuous wave argon ion laser (with prism line selector). Its most intense wavelength is at 488nm and the next is at 515.5nm. These wavelengths are the two lines most commonly used and are suitable for exciting fluorescent dyes such as fluorescein and rhodamine respectively. The advantages of using laser radiation are many;

1. The beam is monochromatic.
2. The beam is coherent (in phase).
3. The laser may be tuned to a number of different wavelengths.
4. The intensity of the beam is stronger than other light sources such as mercury.
5. The beam has a Gaussian transverse energy mode which can be focused to a diffraction limited spot also of Gaussian energy profile ie there is a known intensity distribution at the spot.

Digital Acousto-Optic Modulator (Model 304D, Coherent Associates, Danbury, Conn. U.S.A)

This device is essential for lateral mobility measurements in the apparatus available. It allows both a brief intense laser light for the bleaching phase and a highly attenuated measuring beam for the pre-bleach and recovery phases. This use was first described by Garland (1981). Briefly, the apparatus diffracts the laser beam, and the control system allows it to be on or off. Attenuation of the beam is achieved by changing the on-off time of the duty cycle. Another important fact is that the bleaching and measuring modes differ only in the frequency at which the laser beam is pulsed; and therefore the position of the beam is constant throughout measurement and bleach.

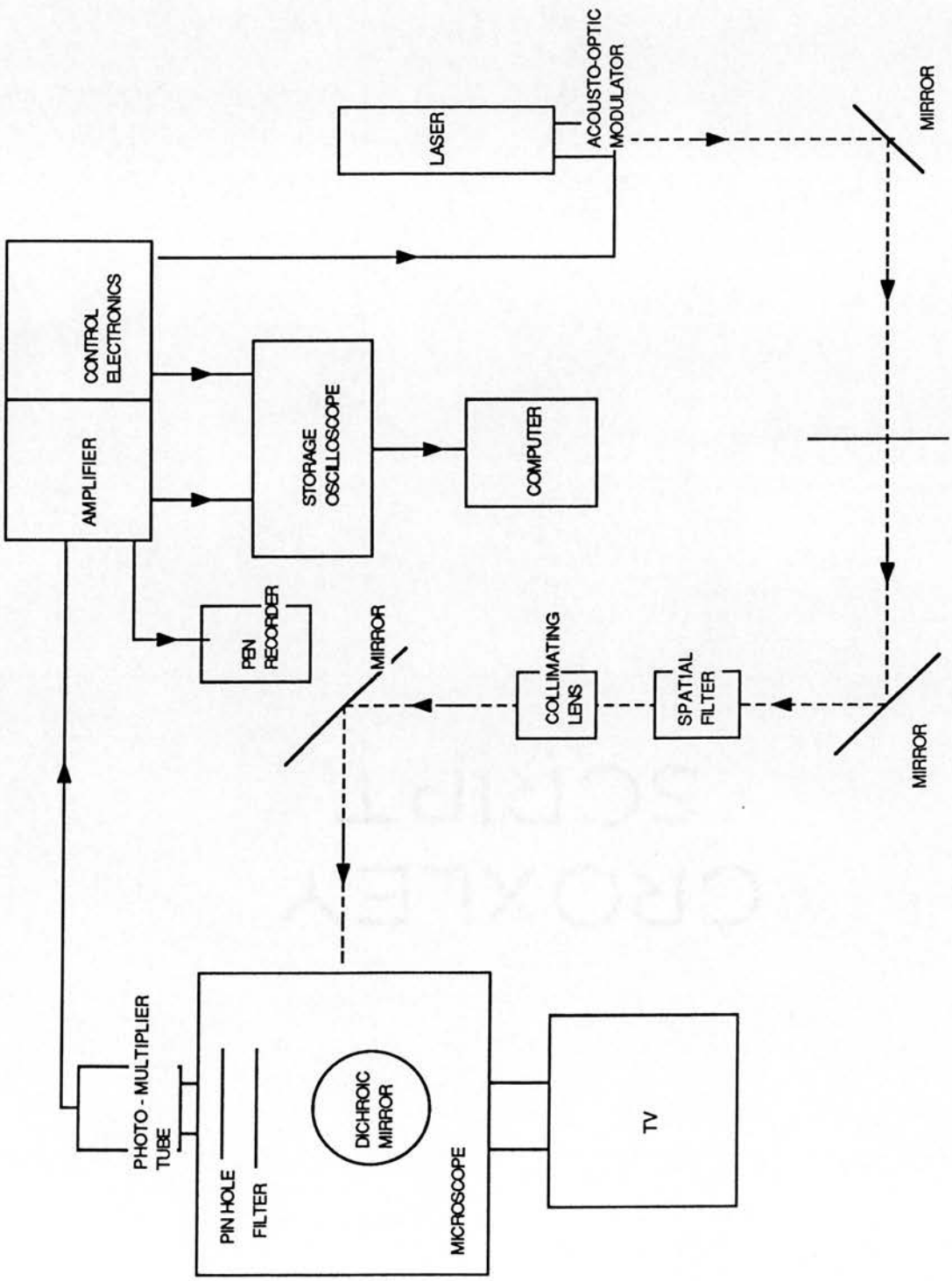


FIG. 3.3 APPARATUS USED TO MEASURE LATERAL MOBILITY

The Spatial Filter (Model 1526 Beam Expander with Spatial Filter, Oriel Scientific, Kingston-upon-Thames, Surrey, U.K.

This equipment consists of a lens and a pinhole and its function is to attenuate the weaker light intensities not associated with the more intense Guassian intensity from the laser output. By doing so it also increases the contrast ratio (ie. the peak to trough intensities of the modulated beam) approximately 10 fold.

Collimating Lens (80mm Focal Length, from Oriel Scientific)

By adjusting the position of this lens, the size of the laser beam at the image plane of the objective (and thus at the sample) could be altered. Thus using a wide beam, the microscope could be used as an ordinary fluorescence microscope. Photobleaching experiments were carried out with a small beam.

The Fluorescence Microscope (Model 41, Vickers, Instruments, York, U.K)

This microscope had been adapted for use with the laser by removal of some of the optics (2 lenses and a diaphragm) to allow laser light to pass in from the back. The laser beam was passed into the microscope and reflected down through the objective onto the sample by a dichroic mirror (Ploem, 1967). Different mirrors may be selected according to the dye used. Thus, if a fluorescein dye has been used to label the cell surface molecules, then a green light reflector would be used such that a beam of 45° incident to the mirror would be 95% reflected at 514.5nm and 80% transmitted above 580nm. Therefore the majority of the laser beam will be reflected onto the sample and the excited fluorescence will be largely transmitted.

The fluorescence then passes through a barrier filter, a 3mm thick non-fluorescent plastic laminate Scholt KV550 filter (H.V. Scan, Solihull, Birmingham, U.K.). This has 100% transmittance at 600nm, 50% at 550nm and 1% at 535nm which gives some idea of its cut off characteristics.

The prism normally present in these microscopes to allow visualisation or photography of the sample was changed so that the laser beam went straight up instead of back to the camera.

The beam then passes through a pinhole, the size of which depends on the size of the laser spot being used and its function is to cut out any background fluorescence. The fluorescence is then detected by a photomultiplier tube (EMI model 9813B) with focusing electrode.

The sample is placed on a thermoelectric X-Y stage (Bailey Inst.) and the incoming beam is focused through a Zeiss X40 water immersion objective.

Optical Components

The whole optical system was arranged on an optical table 1.22 x 0.91m (Oriel, 1220). Extensive use was made of optical rails (Oriel standard rails) and adjustable mirror mounts (Oriel model 1450) which were used to align and direct the laser beam into a 2mm diameter pinhole, the spatial filter and the collimating lens.

The Control Electronics

These were designed and constructed by Peter Garland. The instrumentation consists of;

1. A control for the acousto optic modulator either a) on continuously, b) pulsed (controlled by frequencies) or c) off.

2. Various measuring frequencies.

3. Control of the length of the bleaching pulse (0.1 - 10 secs).

4. A button to trigger the bleaching pulse.

5. An input signal from the Photo Multiplier Tube. This is a current signal and it was converted to a voltage with a current-to-voltage amplifier, followed by a filter of variable time constant (to smooth out noisy traces on pen recorder).

6. An output signal to the pen recorder (model 28000 from Bryans Southern Instruments Ltd., Mitcham, Surrey) or to the Gould-4035 digital storage oscilloscope.

Computer

Hewlett-Packard 85 and 9816 microcomputers were used to store the traces and analyse them by a curve fitting routine based on the Yguerabide (1982) approximation (programmed by John Birmingham). A television camera (JA1 model 732) was used to observe cells position by transmitted light whilst setting up each experiment.

3.5.0 Experimental Procedure

Before each set of measurements was taken under different conditions, a bleach of fluorescent albumin in 90% glycerol, was carried out to check the spot size of the laser.

A day before the experiment 0.08-1.2 x 10^6 cells were seeded onto the 40mm^2 coverslips and left to adhere overnight. All labelling and washing was performed on the coverslip. For examination by FRAP the coverslip was inverted onto a slide with a small well containing cell growth medium (see section 2.2.1). The coverslip was prevented from 'slipping off' by a thin silicon grease seal.

The autofluorescence of unlabelled cells was always measured to check the viability of the cells (dead cells tended to have high autofluorescence values). When the slide containing the labelled cells was placed on the microscope stage, it was left about five minutes for any temperature equilibration to occur. Before cell signal fluorescence was obtained, a measure of the background fluorescence was taken. In general, experiments were only performed if the cell signal to background ratio was at least 10:1.

The TV camera was used to monitor the cells and ensure that the same cell was not measured more than once. The exact position of the measuring beam of the laser could also be observed using the video camera.

3.5.1 Determination of the Spot Size

As can be seen from the equation to calculate the lateral diffusion coefficient, the spot size of laser must be measured accurately as the diffusion coefficient depends on the square of its radius (w^2). The easiest method of measuring the spot size, and one that was carried out before each set of measurements, is that of Thompson and Axelrod (1981).

A simple photobleaching recovery experiment was carried out on a standard sample of fluorescently labelled bovine serum albumin in 90% by wt. of glycerol (see Materials and Methods). This was sandwiched between a microscope slide and coverslip into a layer. As the diffusion coefficient can be calculated theoretically, the spot size can be determined correct to within 10%. The spot size varied very little over a period of several months.

Figure 3.4 (overleaf)

Data traces showing the computer analysed 'best fit' for actual data traces representing:

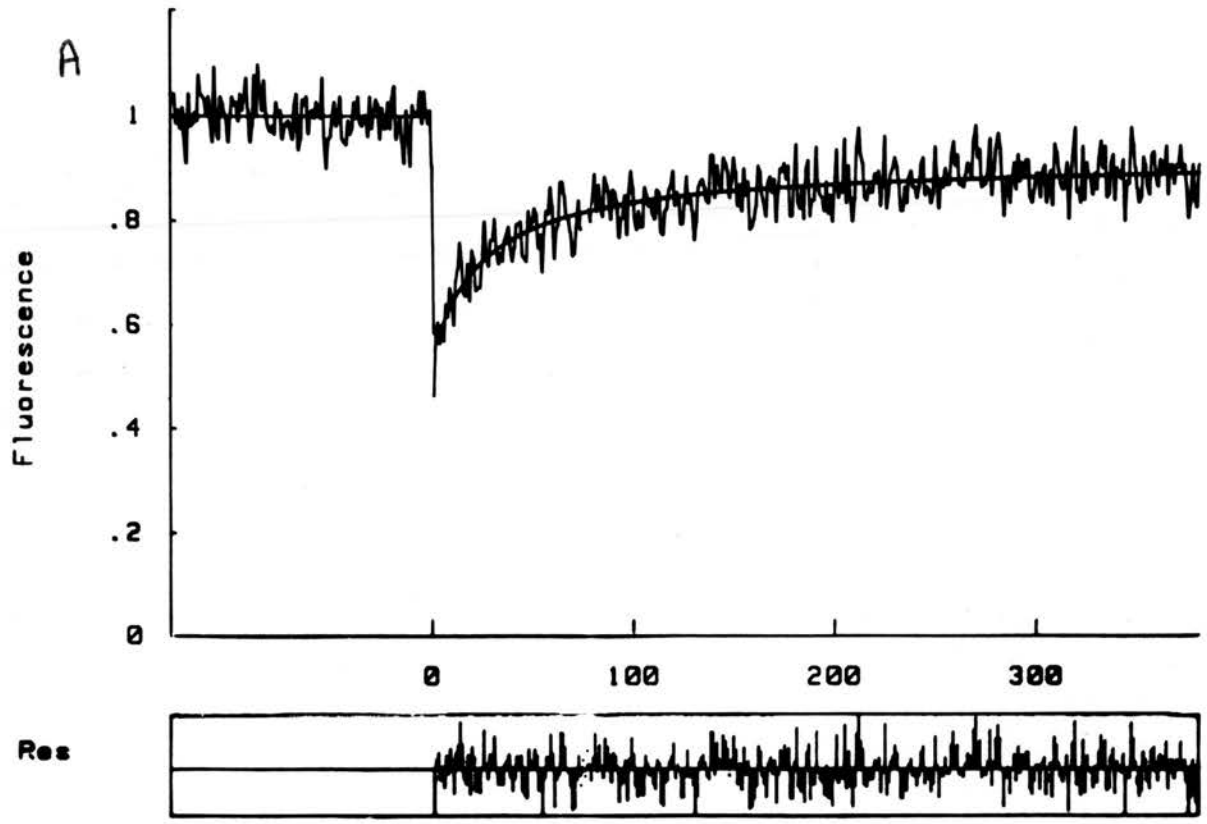
A) Ganglioside GM_1 (where 80% of the molecules observed are mobile)

B) Cholera toxin (where only 25% of the molecules observed are mobile)

FRAP file : GM106

\# points = 380 msec/point = 200

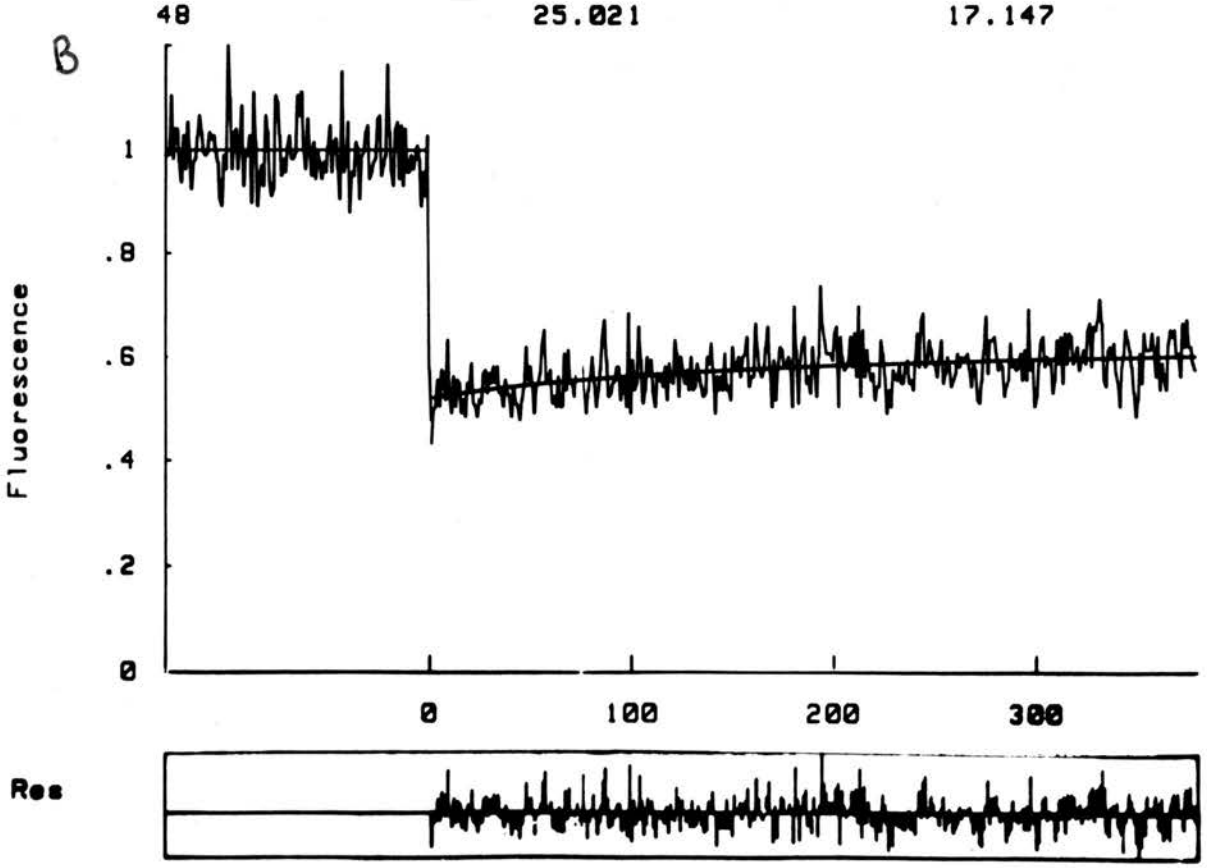
%-bleach	%-recovery	T-half secs
46.55	81.353	5.1644



FRAP file : 24NOV7

\# points = 379 msec/point = 100

%-bleach	%-recovery	T-half secs
48	25.021	17.147



3.6.0 Results of Lateral Mobility Measurements

Fig.2.7 in Chapter 2 (section 2.2.9) shows the concentration dependence of binding of fluorescent toxin against NIH 3T3 fibroblast cells. Technically this experiment was difficult as the laser spot had to be increased and thus the whole FRAP equipment needed realignment. The graph indicates the large variation from two different labelling results. No steady signal was obtainable at a toxin concentration of less than 100ug/ml which is approximately a 1 micromolar solution. The K_m for cholera toxin binding to ganglioside GM_1 is 10^{-9}M so one would expect saturation at a 1um level of toxin. It appears that saturation has not been reached and this suggests that cholera toxin affinity for GM_1 ganglioside has been reduced by conjugation with fluorescein.

3.6.1 Measurements with Fluorescent Cholera Toxin

Despite a few initial technical difficulties, setting up the FRAP equipment and finding the optimal labelling conditions, the FRAP system at Unilever was used to measure the mobility of fluorescent cholera toxin on NIH 3T3 fibroblasts very successfully.

The cells were not labelled with cholera toxin for longer than half an hour, because after this period they tended to become vacuolar either due to the cholera toxin, or lack of nutrients, even if the cholera toxin was diluted in serum free medium. Labelling time was therefore usually fifteen minutes at $4^{o}C$ but coverslips containing labelled cells were placed on a thermostatically controlled slide while FRAP measurements were taken. Incubation of the cells with anything less than 10ug (100ul of 100ug/ml cholera toxin solution) of fluorescently labelled toxin did not produce a good signal to background ratio,

and the traces were very noisy because of the large amplification (gain) needed to observe the signal therefore cells were always labelled with this amount or greater of fluorescent toxin.

Table 3.II shows the diffusion results obtained with fluorescent cholera toxin (either labelled with 100ug/ml fluorescein toxin (F-CT) or 500ug/ml rhodamine labelled toxin (R-CT) as these were the minimum concentrations required to give good signal to noise ratio). There did not appear to be any trend in the lateral diffusion coefficient or percentage mobile fraction of fluorescent molecules (% recovery), if the measurements were done at the pole or centre of a cell (results not shown). Cholera toxin diffused on the cells at a rate which varied from about $6x10^{-11}cm^2s^{-1}$ to $6x10^{-9}cm^2s^{-1}$ with the mobility varying from 17% to 78%. There was a slight (not significant) increase in both the diffusion coefficient and the fraction of mobile molecules with increasing temperature.

Table 3.II

Diffusion results obtained by labelling mouse 3T3 fibroblasts with flourescent cholera toxin

LABEL	TEMP.	NO. OF EXPERIMENTS (NO. OF MEASUREMENTS)	D_L COEFFICIENT (cm^2s^{-1})	% RECOVERY
100ug/ml F-CT	22-24°C	4 (17)	$4.9x10^{-10}$ +/-($4.5x10^{-10}$)	28.9 +/-(11.1)
500ug/ml R-CT	22-24°C	1 (16)	$2.68x10^{-9}$ +/-($1.7x10^{-9}$)	56.17 +/-(8.54)
500ug/ml R-CT	37°C	1 (12)	$4.2x10^{-9}$ +/-($2.1x10^{-9}$)	71.09 +/-(7.1)

F-CT=Fluorescein labelled toxin
R-CT=Rhodamine labelled toxin

D_L = Lateral diffusion coefficient
% Recovery = percentage of mobile molecules

Coverslips containing cells (0.08-1.2 $x10^6$) were labelled with the specified toxin concentration at 4°C for fifteen minutes. FRAP measurements were taken after 5 minutes temperature equilibration of the slides containing coverslips.

3.6.2 Measurements with Fluorescent GM_1 Ganglioside.

Inserted GM_1 (fluorescently labelled) produced a punctate labelling of cells (a visual indication of ganglioside clusters). The percentage recovery obtained varied from about 60%-80% and the lateral diffusion coefficient was about $1.2 \times 10^{-9} cm^2 s^{-1}$.

A labelling period of half an hour at 37^oC was chosen and kept to as this was a commonly used time period in previous ganglioside mobility experiments. Further experiments were also performed to examine the effect of adding unlabelled cholera toxin followed by one or two antibody layers to cells labelled with inserted fluorescent ganglioside or unlabelled ganglioside when the second antibody was fluorescently labelled instead. Addition of cholera toxin (1mg/ml) was for 10 min at 4^oC. The diffusion coefficient and percentage recovery of fluorescent ganglioside were not significantly altered by addition of cholera toxin (about $8.8 \times 10^{-10} cm^2 s^{-1}$ and 52%-71% respectively). The polyclonal rabbit anti-cholera toxin was diluted (1/250) in PBS and left on the coverslip prior to FRAP measurement. Addition of the 1st antibody also had little affect on the diffusion coefficient and percentage recovery of the fluorescent ganglioside ($2.4 \times 10^{-9} cm^2 s^{-1}$ and 40-60%). The goat anti-rabbit antiserum diluted (1/50) in PBS from Miles Scientific was also left on coverslip prior to FRAP measurement. This second antibody did not seem to alter the diffusion coefficient ($7 \times 10^{-10} cm^2 s^{-1}$) but the percentage recovery was slightly lower (30-42%). See table 3.III for tabulated results.

Table 3.III

Diffusion results obtained by labelling mouse 3T3 fibroblasts with exogenous flourescent GM_1 ganglioside alone followed by cholera toxin, rabbit anti-cholera toxin antibody and goat anti-rabbit antibody.

LABEL	TEMP.	NO. OF EXPERIMENTS (NO. OF MEASUREMENTS)	D_L COEFFICIENT (cm^2s^{-1})	% RECOVERY
100ug/ml F-GM_1	37°C	4 (47)	$1.2x10^{-9}$ +/-($1.0x10^{-9}$)	71.6 +/-(12.7)
100ug/ml F-GM_1 +cholera toxin	37°C	3 (33)	$8.8x10^{-10}$ +/-($5.6x10^{-10}$)	62.8 +/-(9.6)
100ug/ml F-GM_1 +cholera toxin +1st antibody	37°C	1 (8)	$2.4x10^{-9}$ +/-($1.2x10^{-9}$)	51.0 +/-(11.1)
1/50 F-2nd antibody GM_1 +cholera toxin +1st antibody	37°C	1 (5)	$7x10^{-10}$ +/-($5.0x10^{-10}$)	37.46 +/-(7.2)

F=Fluorescein labelled

cholera toxin labelling (1mg/ml) for 10mi

1st antibody = rabbit anti-cholera toxin added 1/250 dilution in PBS

2nd antibody = goat anti-rabbit antibody added 1/50 dilution in PBS

3.7.0 Discussion

Both bound cholera toxin and inserted ganglioside lateral diffusion measurements were obtained. Surface micelles of fluorescent ganglioside or aggregates of fluorescent cholera toxin can be excluded from the observations made as such assemblies do not fluoresce (Skiar et al., 1980).

When cholera toxin bound to NIH 3T3 mouse fibroblasts 17%-78% of the fluorescent toxin molecules were mobile after binding to the cell surface. The lateral diffusion coefficient of the cell bound toxin was about 1 x $10^{-9}cm^2s^{-1}$.

Inserted fluorescein-labelled GM_1 ganglioside had about 60%-80% mobility and a diffusion coefficient 1.2 x $10^{-9}cm^2s^{-1}$ in the same type of cell.

From these results one can say that addition of cholera toxin to cells containing inserted GM_1 ganglioside appeared to have no effect on the mobility of the inserted ganglioside. This observation holds true if one measures the cholera toxin or the ganglioside mobility. Further addition of a primary antibody against the cholera toxin did not change these values of mobility for inserted ganglioside. However addition of a secondary antibody reduced the fraction of mobile molecules (antibody) to 37%. As the measured species on addition of second antibody was the second antibody and not the ganglioside, this might indicate that not all ganglioside species were labelled by the additions and a fuller picture could have been obtained had it been possible to label both the ganglioside and the antibodies and follow their mobility concurrently.

Therefore, the mobility of cell bound cholera toxin appeared to be similar to the mobility of inserted ganglioside in terms of the percentage of mobile molecules and the lateral diffusion coefficient. A logical extension of these observations is that binding of cholera toxin to *in situ* cell ganglioside GM_1 does not appear to alter the number of mobile ganglioside molecules or the rate at which they diffuse, if one uses inserted gangliosides as a model for *in situ* ganglioside mobility.

One might expect a potentially pentavalent molecule such as cholera toxin to cross link five ganglioside molecules and for this complex to be more immobile than one ganglioside alone. However, it has been shown by Saffman and Delbruck (1975) that the size of the macromolecular complex moving through the viscous lipid membrane has a small effect on mobility (less than 5% decrease in mobility if size is increased 5 fold). It is the viscosity of the medium the molecular species is moving through that has a greater influence on mobility.

Therefore it is not reasonable to assume that a crosslinked toxin-ganglioside complex would have a slower lateral diffusion coefficient than one ganglioside molecule alone unless for some reason one or more of the gangliosides linked are immobile.

3.7.1 Variation in Results

The standard deviation for the lateral diffusion coefficient values were high but they were of the same order of magnitude obtained by other workers working on FRAP measurements of biological specimens (Dragsten et al., 1979, Foley et al., 1986).

The variations obtained might have occurred due to effects of the passage number of the cells measured and therefore their age, the amount of adherence of individual cells to the glass surface or perhaps the state of growth of the cell, as these results were obtained for different cells during an experiment and from different experimental days. Changes in the mobility of the major histocompatibility antigens were observed in fibroblasts with increasing age of cell culture, increased cell contact and with increased extracellular matrix material (Weir and Edidin, 1986). As a result of this variation only gross differences in the rate of diffusion or the mobility of the measured ligands can be identified from such measurements.

Another important factor to consider regarding a possible contribution to variation in the results obtained is the toxin-ganglioside ratio. The exact amount of toxin bound to ganglioside for each set of measurements was not determined but the maximum toxin-ganglioside ratio could be calculated from the known amount of GM_1 molecules on the cell surface (1×10^6) assuming all the toxin available had bound.
This ratio always favoured the toxin, which in all experiments was at least in twenty fold excess of the total ganglioside present and at most in one hundred fold excess assuming all the toxin added, bound to the cell. This high ratio was necessary to obtain sufficient labelling of the cells to be detected by a photomultiplier tube. This is supporting evidence together with the binding concentration dependence of fluorescent toxin, that the binding affinity of the toxin had been lowered when fluorescein was added. Therefore, the exact stoichiometric ratio of bound toxin to ganglioside was not known. In experiments with inserted ganglioside the amount of ganglioside inserted was not determined. Therefore one can only assume that the data may represent toxin bound to one or more ganglioside molecules (five being the maximum).

Perhaps the thirty per cent of immobile toxin molecules represents those toxin molecules which are bound to GM_1 which is not free to move. Inserted ganglioside shows a similar degree of immobility, being about thirty per cent. Maybe thirty percent of the cell surface is immobilised due to high protein concentrations and/or the presence of cytoskeletal attachments.

3.7.2 Possible Restraints to GM_1/ Cholera Toxin Lateral Diffusion in the Plasma Membrane.

Although the initial lateral diffusion measurements made were for membrane proteins, many studies have examined the lateral diffusion of lipids in cell membranes. The typical diffusion coefficient for a lipid is in the order of $10^{-8}cm^2s^{-1}$ (Peters, 1988).

However in artificial membranes, lipids move 10 times faster, indicating that they are restricted in cellular membranes. This restriction of both protein and lipid lateral diffusion in cell membranes may indicate something about their interaction in the membrane. Peters (1981) states that a simple explanation for the restriction of lipid mobility might be geometric hindrance by proteins or interactions with lipoproteins.

The data presented in table 3.0 on the capping observed with cholera toxin receptor complexes suggests that the lateral diffusion of ganglioside GM_1 maybe regulated by transient interactions between transmembrane integral proteins and cytoskeletal elements.

Experimental evidence is available to show that a glycolipid, possibly a ganglioside is tightly associated with the protein b_1-adrenoreceptor in turkey erythrocytes (Sinai et al., 1986) and the experimenters state the generality of this finding. That gangliosides have been found closely associated with receptors suggests that they may function in trans-membrane signalling or receptor maintenance and stabilisation within the bilayer.

In my experiments a population of immobile ganglioside molecules existed (30% of inserted ganglioside) and this may be because they became associated with proteins linked to the cytoskeleton or to other in situ gangliosides which were immobilised by skeletal linkage. This possibility of ganglioside association is discussed in the following section.

3.7.3 Ganglioside Clusters

The following evidence from lateral diffusion measurements supports the hypothesis that gangliosides themselves may be clustered. When cells were labelled with fluorescent gangliosides the labelling appeared punctate also indicating clustering. The majority of gangliosides are mobile but some are immobile, and the binding of the cholera toxin might mimic this distribution. This is not an unreasonable suggestion as the effect of the crosslinking second antibody could be explained in this context.

The effect of the second antibody would be to cross link cholera toxin molecules which have at least one immobile ganglioside bound and are thus immobile with cholera toxin molecules which are bound to up to five mobile gangliosides.

This crosslinking would effectively immobilise the whole complex and thus could account for the 50% reduction in mobile molecules upon addition of the crosslinking second antibody. Fig. 3.5 indicates three of the possible binding combinations of cholera toxin with ganglioside, which cannot be discounted by the data.

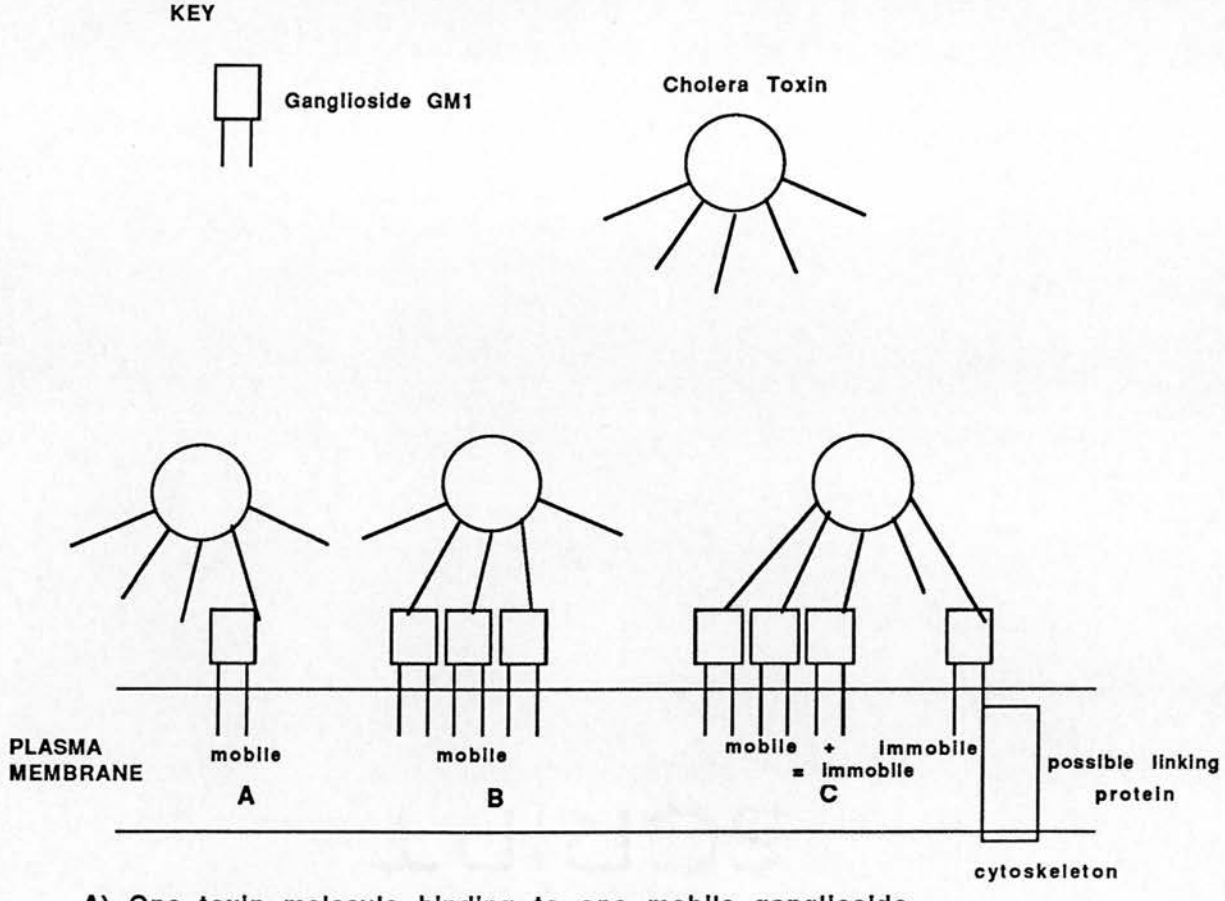
Another hypothesis which is not inconsistent with the results is that the immobile ganglioside clusters may be in distinct regions on the cell surface. Because, if they were heterogeneously distributed about the cell surface the data would show a greater fraction of immobile molecules for a given number of immobile gangliosides.

The idea of gangliosides forming clusters on the cell surface is not a new one. Spiegel et al. (1984) suggested that gangliosides existed on the plasma membrane as clusters. She showed that in lymphocytes, addition of anti-rhodamine antibodies capped both rhodamine and lucifer yellow labelled gangliosides present in the membrane. Similarly, cholera toxin capped GM_1 and GD_{1a} gangliosides, to which it does not bind indicating that gangliosides may be associated with each other.

Sharom and Grant (1978) using the Electron Proton Resonance (EPR) spectroscopy technique found that gangliosides exhibited strong carbohydrate attraction by virtue of the hydrogen bond formation between carbonyl groups. This fact might explain the immobility of 40% of the gangliosides. The immobile ganglioside molecules could also be associated with a protein molecule. These results therefore indicate that the plasma membrane of fibroblasts is not homogeneous and that distinct regions of lipids occur within it.

FIG 3.5

1) Models of Cholera toxin binding to Ganglioside GM1



A) One toxin molecule binding to one mobile ganglioside

B) One toxin molecule binding to more than one mobile ganglioside

C) One toxin molecule binding to a mixed population of mobile and immobile gangliosides

In relation to cholera toxin action, it might be the toxin interaction with the immobile fraction which is important in the further sequence of events of toxin action i.e. insertion of the A_1 active polypeptide. Furthermore, immobilisation of cell surface constituents by addition of a cross-linking second antibody has previously been shown in the membrane of E. coli by Davison and Garland (1983), indicating again regions of mobile and immobile molecules.

3.7.4 Comparisons of Lateral Diffusion of GM_1 Ganglioside with Previous Measurements

The lateral diffusion coefficient of $1.2 \times 10^{-9} cm^2 s^{-1}$ for fluorescent GM_1 ganglioside compares very well with previously published results of mobility measurements of ganglioside. It is within one order of magnitude of the other results.

In these experiments the percentage of mobile ganglioside molecules was never 100%. However, when Spiegel et al., (1984) performed a similar experiment in a primary culture of human foreskin fibroblasts, she found that all the ganglioside molecules were mobile. The difference in mobility might reflect the difference in density of the cells, as she also found a correlation between higher cell density and immobility, or it might just reflect a difference in the organisation of the plasma membrane of the two fibroblast cell lines. Previous results also indicated a generally greater mobile fraction of between 90 and 100% for ganglioside GM_1 which again might reflect differences in the plasma membrane.

3.7.4.1 Are Inserted GM_1 Molecules a Good Model for in situ Ganglioside Molecules?

The evidence that inserted fluorescent GM_1 derivatives do function in the same way as in situ GM_1 molecules was presented by Spiegel (1985). She showed that these inserted derivatives were able to function as receptors for cholera toxin in cells which had an adenylate cyclase enzyme but no GM_1 molecules. After insertion of the ganglioside the cholera toxin bound to the cell and activated the adenylate cyclase, which it did not do on untreated cells. There is much more evidence to suggest that GM_1 molecules act like in situ gangliosides (see section 1.2.4.7).

In principle a better measurement of lateral diffusion of in situ gangliosides would have been to use a monovalent ligand eg. fluorescent Fab antibody specifically directed against GM_1. In practice, however, most antibodies which react with GM_1 will cross-react to some extent with other glycolipids and glycoproteins and a specific antibody was not obtainable for these experiments.

CHAPTER 4

EXAMINATION OF THE CHOLERA TOXIN RECEPTOR BY IMMUNOGOLD LABELLING; OBSERVATION OF A POSSIBLE NUCLEAR LOCATION OF GM_1 GANGLIOSIDE IN MOUSE SMALL INTESTINAL CELLS

CHAPTER 4

EXAMINATION OF THE CHOLERA TOXIN RECEPTOR BY IMMUNOGOLD LABELLING; OBSERVATION OF A POSSIBLE NUCLEAR LOCATION OF GM_1 GANGLIOSIDE IN MOUSE SMALL INTESTINAL CELLS.

4.0 Introduction

In this chapter the first observation of a cholera toxin binding site (probably ganglioside GM_1) within the heterochromatin of the nucleus of mouse small intestinal cells is described.

After exploring the mobility of ganglioside GM_1 on the cell surface by lateral diffusion I wanted to see whether this receptor could be found inside a cell and where it was located intracellularly. To answer these questions the technique of immunogold labelling was used.

To locate the ganglioside GM_1 cholera toxin and an anti-ganglioside GM_1 antibody were used as the probes. First a gold-toxin probe was prepared. Direct observation under the electron microscope was used to check that toxin was adsorbed to the gold and using an easily grown cell line (NIH 3T3 mouse fibroblasts) it was confirmed that the toxin-probe bound to the cell surface and was internalised. This test showed that the toxin had retained its binding properties after combination with the gold. My aim was to use this toxin-probe on fixed, embedded mouse small intestinal tissue to locate the intracellular position of the ganglioside GM_1. Small intestinal tissue was chosen because it is the classical target cell of the toxin. However, the toxin-probe did not bind to the embedded tissue especially on the plasma membrane, an area known to be rich in ganglioside GM_1.

The possible reasons for this lack of binding are discussed later. Therefore, in order to locate the GM_1 in the embedded tissue a sandwich of probes was used consisting of cholera toxin followed by rabbit anti-toxin and gold labelled goat-(anti-rabbit) antibody to probe for intracellular GM_1 sites within mouse small intestine.

As background to this chapter a brief historical perspective of the immunogold technique is given as well as a summary of the properties which make it a very powerful tool in cell biology. Then the materials and methods used are detailed, followed by the results obtained. Finally the results are discussed and related to what is already known about cellular GM_1 ganglioside location and action.

4.1.0 Background

4.1.1 Historical Perspective -Immunocytochemistry and Immunogold

The aim of cytochemical techniques is to localise specific biochemical components in particular tissue and cell compartments. In 1941, Albert H. Coons initiated 'immunocytochemistry'. He employed antibodies which were directed against cell components and were conjugated to a fluorescent molecule. These were the first experiments to observe the localisation of cellular components by eye (Coons, 1941). Since then antibodies have also been conjugated to enzymes (Nakane and Pierce, 1966). The use of an enzyme allowed visualisation of the antibody location because after a suitable enzyme-specific substrate had been added, a dense or coloured reaction product was left.

Essentially two main types of marker exist in cytochemistry. These are non-particulate or particulate. Horse-radish peroxidase (an enzyme) is an example of a non-particulate marker. Radioisotopes are also non-particulate markers. Non-particulate markers are generally sensitive because they can "amplify" the identification since it is easy to detect their presence. However precise localisation (especially under the electron microscope) is made more difficult because of the diffuse signal given (Varndell and Polak, 1986). Particulate markers such as ferritin or gold are preferred for the precise localisation and quantification of cellular components. This is because one observes their position, rather than their presence.

4.1.2 Colloidal Gold

Gold particles are negatively charged and they remain stable in solution by virtue of electrostatic repulsion. However, if electrolytes such as sodium chloride (Na^+Cl^-) are added to a colloidal gold solution, the ionic layers around the gold are compressed and the particles approach one another.

At a critical distance the particles cohere and flocculation occurs. In 1901, Zsigmondy made the observation that this flocculation of gold was prevented by addition of protein solutions. This observation is the basis for the current use of colloidal gold coated to a large variety of biochemical ligands in immunological studies.

Thiessen (1942) not only proved the particulate nature of colloidal solutions of gold by electron microscopy but he also observed that proteins adsorbed to the gold formed layers around the gold particles. Geoghegan and Ackerman (1977) found that if a protein was added to gold at the pH of its isoelectric point when the Zwitterion ion form (containing both positive and negative charges) was predominant, good stable adsorption took place. This fact is still widely accepted in formation of gold-protein conjugates. Some recent work however challenges the belief that gold adsorption must be carried out at the isoelectric point of the protein. De Roe et al. (1987) discovered that, for some proteins, adsorption of the protein to the gold was independent of the isoelectric point of the protein.

The colloidal gold marker system is now widely used because of the following advantages:

1) Gold markers are very electron dense and have a characteristic shape, which means that they are very easily identified and cannot be easily confused with biological structures.
2) Gold markers are fairly easily prepared, retain most of the binding properties of the protein/antibody adsorbed and are stable for a long time.
3) Gold markers may be used both at the light and electron microscope level.

If a colloidal gold solution consists of spherical particles with a diameter less than 80 nm, then it is usually a red colour in transmitted light.
Colloidal gold appears blue if it contains larger particles (> 80m), aggregated particles or non spherical particles.

It was not until 1971 that colloidal gold was applied in immunological work with the introduction of 'an immunocolloid method for the electron microscope' by Faulk and Taylor (1971). In their experiment, rabbit anti-salmonella antiserum was adsorbed onto gold particles and then incubated with salmonella bacteria. The surfaces of the bacteria were specifically labelled. Since this early experiment many other direct labelling experiments have been carried out.

Since 1977, very many ultrastructural localisation studies have been carried out using colloidal gold adsorbed to a variety of ligands such as immunoglobulins, lectins, toxins and protein A. The following reviews give a more detailed account of the use of colloidal gold as a marker: (Horisberger, 1981; Goodman et al., 1981; Roth, 1982, 1983).

4.1.3 Pre-embedding and post-embedding immunogold

A protein-gold conjugate can be added to a biological preparation before or after it has been processed and embedded in resin ready for electron microscopy. Which of these two possibilities is used makes a major difference to the results and to their interpretation. For a detailed review of both these methods see the review by Beesley (1985).
Pre-embedding immunolabelling involves detection of the antigen by the antibody (or receptor by the ligand) immunologically before the tissue is processed and embedded in resin for electron microscopy. On the other hand, post-embedding involves immunological detection on sections of tissue which have already been processed, embedded in resin, sectioned and placed on grids for electron microscopy.
The pre-embedding technique is usually used for detection of external cell surface antigens. Post-embedding allows for antigen localisation internally or externally, but not all antigens survive the tissue fixation procedures used. The number of antigens exposed to the antibody will also depend on how the tissue section has been cut.

In my experiment to see whether the gold toxin probe I had made bound to NIH 3T3 cells I used the pre-embedding technique, whereas in trying to locate intracellular GM_1 sites I made use of post-embedding immunogold.

4.2.0 Materials and Methods

Materials

The reagents used for experiments described in this chapter were obtained from the following sources:

MATERIAL	SOURCE
Double distilled water (HPLC grade)	Rathburn Chemicals Ltd. Walkerburn, Scotland
2% Solution of dimethyl-dichlorosilane in 1,1,1 trichloroethane (for production of a silicone surface on glass) 'REPELCOTE'	Hopkin and Williams, Chadwell Heath, Essex, England
Coverslips (20mm x 20mm) Slides	Chance Propper Ltd., Sponlane, Smithwick, Warley, West Midlands England
Osmium Tetroxide Gelatin capsules	Taab Laboratories and Equipment Ltd.,3 Minerva House, Calleva Industrial Park, Aldermaston, England
Nickel Grids	Agar Scientific, 66a Cambridge Road Stanstead, Essex, England
LR Gold	London Resin Co., PO Box 29, Woking, Surrey, England

GMA (Glycol Methacrylate) 0.01% Benzoin ethyl ether	Polysciences Inc., 24 Low Farm Place, Moulton Park, Northampton, England
Melanex	ICI Chemicals and Polymers Ltd., PO Box 13, The Heath, Runcorn, Cheshire, England
Collodion	Specially prepared at Unilever Laboratory, Colworth House, Sharnbrook, England
Gold labelled anti-rabbit antiserum (15nm)	Janssen Life Science Products, Olen, Belgium (now part of Amersham International)
Rabbit anti-cholera toxin antiserum	Gift from Dr. S. van Heyningen (van Heyningen, 1976)
Rabbit anti-ganglioside GM_1 antiserum	Gift from Dr N. Gregson, Dept of Anatomy, Guys Hospital Medical School, London
B subunit of cholera toxin	Separated from whole toxin by gel filtration on Sephadex G77 column in 5mg/ml 6.5M urea and 0.1M glycine pH3.2 (T.Scobie Ph.D thesis, 1986)

All other inorganic reagents were analytical grade from BDH and the biochemicals came from Sigma.

4.2.1 Methods

4.2.1.1 Preparation of Colloidal Gold

Glassware used in colloidal gold preparation must be scrupulously clean as contaminants can interfere with formation of the colloid, causing variation in the particle size. Therefore, after thorough cleaning, glassware must be rinsed in double distilled, filtered water and then covered in silicon (Repelcote). All the reagents are also made up in the double-distilled ultra pure water.

4.2.1.1a The method of Frens (1973)

There are several methods of preparing colloidal gold but this method was used in preference to the others because it is the only one which produces gold particles of a uniform size. It was used to produce 16nm diameter gold particles. The size of the particle produced by this method depends upon the volume ratio of sodium citrate (1% w/v aqueous solution) to tetrachloroauric acid (0.001% w/v solution). The greater the volume of sodium citrate added, the bigger the gold particle is.

One hundred millilitres of a 0.01% (w/v) tetrachloroauric acid solution was heated to boiling and 4ml of 1% (w/v) trisodium citrate aqueous solution was then added quickly. After about five minutes of gentle boiling the colloid was produced and the appearance of a reddish-orange colour indicated that the reduction was complete.

4.2.2 Complex Formation of Colloidal Gold with Cholera Toxin

The adsorption of protein onto gold particles is still not a well understood phenomenon (De Roe et al., 1987; Horisberger, 1981; Goodman et al., 1981). Roth (1983) states that it is generally assumed that the proteins adsorb onto the gold surface due to electrostatic interactions between positive groups on the protein and the negative surface charges on the gold particles.

Before adsorption, one must first determine the minimum amount of protein required to stabilise the gold. This is especially important if the protein is precious! The general method is identical to that followed by Roth and Binder (1978) who mixed 0.5ml of colloidal gold with 0.1ml of a serially diluted protein. After about one minute, 0.1ml of 10% aqueous sodium chloride solution was added and the stabilisation effect was judged by eye, looking at the colour of the colloidal gold. The lowest protein concentration which maintained the red colour of the solution after the NaCl addition was taken as the amount to be used, usually with an extra 10% added to ensure stability (Geoghegan and Ackerman, 1977).

4.2.2a Formation of Colloidal Gold-Cholera Toxin

The method of Geoghegan and Ackerman, 1977, was followed. Montesano et al. (1982) had previously made colloidal gold-cholera toxin and had determined the isoelectric point of cholera toxin as pH 6.9.

Therefore, after 16nm colloidal gold solution had been made by the method of Frens (1973) (see section 4.2.1.1a), the pH of the solution was adjusted to 6.9.

In order to do this a measured aliquot of colloidal gold solution was taken and a few drops of 1% aqueous polyethylene glycol were added to prevent the colloidal gold solution from plugging the pores of the pH electrode (Geoghegan and Ackerman, 1977). Then drops of 0.2M K_2CO_3 (potassium carbonate) were added to bring the pH to 6.9. The amounts are then scaled up for the total volume of colloidal gold solution.

Then 0.5ml of pH-adjusted colloidal gold solution was taken and 0.1ml of a dilution of cholera toxin (15 ug/ml - 100 ug/ml) was added and the mixture left for one minute. Then 0.1ml of 10% sodium chloride solution was added and left for 3 minutes before the solutions were observed by eye and the absorbance measured at 580nm to check the minimum dilution of protein which prevented the gold from clumping into large masses. Cholera toxin at a concentration of 35 ug/ml was the minimum amount required to stabilise the colloidal gold. See fig. 4.1a, which shows the curve generated from a series of absorbance readings. The point where the curve becomes asymptotic with the x axis was taken as the minimum quantity of protein needed to stabilise the gold. This figure was rounded up to 40 ug/ml just to be certain that enough protein was present. Thus the minimum protein (cholera toxin) required per ml of colloidal gold solution was about 8 ug. The observation that a weight of 4ug (0.1 ml of 40 ug/ml solution) stabilised 0.5 ml gold implies that 8ug will stabilise 1 ml of the colloidal gold solution.

Therefore at least 800ug of cholera toxin was required to stabilise 100ml of colloidal gold which had been pH adjusted to 6.9 (isoelectric point of cholera toxin). The 1mg batch in which the toxin was supplied was dissolved in water and added slowly to 100ml of the colloidal gold solution while it was being gently stirred.

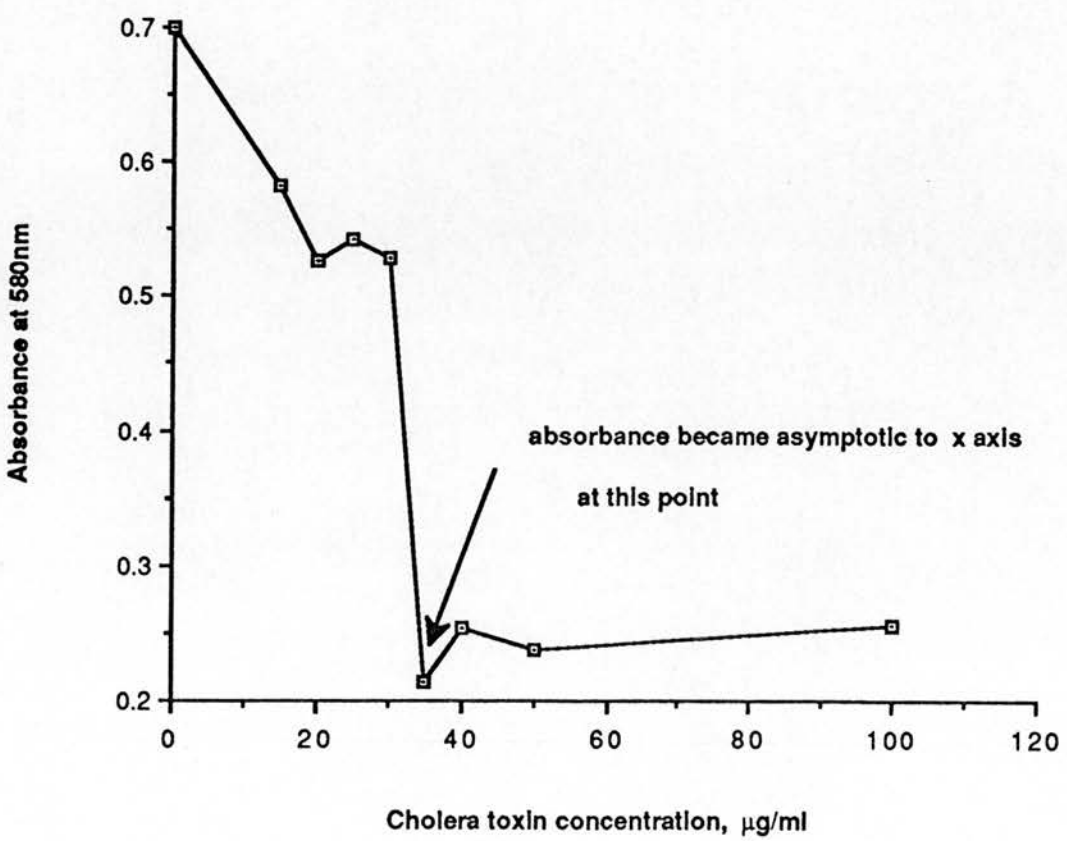
Some of this solution was dried onto an electron microscope grid and lightly counterstained in 2% aqueous uranyl acetate before being observed by electron microscopy to see if the protein had been successfully adsorbed onto the gold (see fig. 4.1b). Removal of free uncomplexed protein was achieved in the following way. The complex was divided into two 50ml batches and balanced, then given a low speed spin (1500 revolutions per minute (rpm)) in a Beckman L2-75B ultracentrifuge to remove any gold aggregates which had formed.

The supernatant was taken and recentrifuged for an hour in the same centrifuge at 11 500 rpm to separate the colloidal gold-protein from uncolloided protein. This was repeated to ensure that all the free protein was removed. Each time the dense red sediment was taken up in PBS/0.2% Polyetheylene Glycol (PEG). The final volume of concentrated gold-toxin probe was 10ml.

4.2.3 Storage and Stability of Complexes

The complexes were stored at $4^{o}C$ with addition of 0.001% sodium azide to prevent bacterial contamination. It has been shown that nearly all proteins so far adsorbed onto gold have maintained their binding or enzymic properties (Bauer et al., 1975). The exception has been beef liver catalase, which was completely inactivated by the adsorption process (Horisberger and Rosset, 1977). Roth (1982) gives evidence for good stability of most proteins with gold, but states that the complex should be used within a short time after preparation or centrifuged before use to remove free protein which might have been released into the buffer.

Figure 4.1a Shows the curve generated from a series of absorbance readings of tubes containing 0.5 ml of colloidal gold (with 0.1 ml of a 10 % sodium chloride solution added) against cholera toxin concentration present in the tubes. The point where the curve becomes asymptotic to the x axis was taken as the minimum quantity of protein needed to stabilise the gold.



4.2.4 Determination of Binding Properties of Colloidal Gold - Toxin Probe

4.2.4.1 Experimental Procedure

NIH 3T3 cells were plated on plastic (ICI Melanex) coverslips, one day prior to use. Some cells were then labelled at 37°C with 1ml of colloidal gold-cholera toxin conjugate at 1/50 dilution in PBS. Other cells were labelled with the same concentration of gold conjugate after fixation in 2% glutaraldehyde/0.5% paraformaldehyde in PBS pH 7.5. After 30 min incubation all cells were further fixed for fifteen minutes in the same fixative. Additional coverslips acted as controls where an excess of unlabelled toxin was added to the cells (1 ml of 1 mg/ml toxin) before fixation and addition of gold labelled toxin. The coverslips were then dehydrated through a series of increasing alcohol concentrations (50% to 100%) and placed longitudinally in a gelatin capsule where they were left overnight in a resin mix (4 volumes LR gold to 1 volume GMA containing 0.01% benzoin ethyl ether). The resin was renewed and polymerised at room temperature for 24 hrs under a U.V. light. The resin blocks were prepared for cutting with a glass knife; then thin sections (80-100nm) were cut with an ultramicrotome (Sorvall MT-2) using a diamond knife (Dupont Ltd.), and mounted on nickel grids. The cell sections were examined with a Jeol 100CX 2 electron microscope with an accelerating voltage of 80KV.

4.2.5 Examination of Internal and external GM_1 Sites by Post-Embedding Immunogold Labelling

4.2.5.1 Experimental Procedure

4.2.5.1.1 Tissue Preparation

An adult F_1 C57B x CBA mouse was sacrificed by cervical dislocation and the small intestine removed. The tissue was then fixed in freshly prepared 1% paraformaldehyde/0.5% gluteraldehyde in PBS for 2 hours at 4°C. As much of the gut contents were removed as possible by forcing them out with a 'jet' of PBS. The tissue was then cut into small transverse sections and these were dehydrated through graded alcohols (fifteen minutes in 50% through to absolute alcohol). The tissue was then embedded in hydrophilic resin block (3 parts LR gold, 2 parts glycol methacrylate (GMA) low acid 0.01% benzoin ethyl ether). The blocks were polymerised for 24 hr at room temperature by U.V. irradiation. Sections were cut using a Sorvall MT-2 ultramicrotome with a diamond knife (Dupont Ltd.) and mounted onto nickel grids.

4.2.5.1.2 Immunolabelling Procedure

All incubations were done in a moist chamber. Collodion-coated nickel grids with the attached thin sections were placed on a droplet of 0.5M ammonium chloride at room temperature for one hour. This is important to block any free aldehyde groups exposed from the fixation which are reactive with ϵ-amino groups on proteins. Then the grids were washed with a spray of PBS and placed on a 1% - 3% ovalbumin solution in PBS for 30 mins at 37°C as a further blocking procedure.

Grids were then transferred onto 10ul diluted drops of colloidal gold toxin conjugate (1/50 to 1/500) or 10ul drops of 850ng/ml cholera toxin diluted in the ovalbumin solution and incubated for 30 min at 37°C. The cholera toxin dilution (850ng/ml) was that used by Hansson et al., (1977) which also gave intense specific labelling and a low background. The antibody concentrations were chosen in the same manner.
The grids were then 'jet' washed gently with PBS sprayed from a Pasteur pipette several times. After washing, the grids were placed section down on a 10ul drop of 1/250 rabbit anti-(cholera toxin) serum diluted in PBS and incubated for one hour at 37°C. At this stage in some experiments, grids were labelled with a 1/50 dilution of anti-GM_1 antibody.
Experiments were also carried out using both antisera to examine whether one would block the other. The washing procedure was repeated and the grids were then incubated with gold labelled goat anti-(rabbit IgG) antiserum at 1/50 dilution in PBS for 30 min at 37°C.

The grids were again washed in PBS as before and further washed in distilled water. Counterstaining of the sections was carried out for 5 min in 2% aqueous uranyl acetate followed by 1 min in lead citrate at room temperature.

The next page illustrates the binding steps in a flow chart.

FLOW CHART SHOWING THE DIFFERENT BINDING STEPS USED TO IDENTIFY CHOLERA TOXIN RECEPTORS IN SMALL MOUSE INTESTINE

Thin section on collodion coated nickel grid

|

|

Placed on 0.5M Ammonium Chloride aqueous solution

22^{o}C for one hour then washed in PBS

|

|

|

Placed on 1-3% Ovalbumin in PBS solution

37^{o}C for 30 minutes

|

|

Placed on 10 ul drop of 850 ng/ml cholera toxin diluted in 1-3% ovalbumin diluted in PBS

37^{o}C for 30 minutes then washed in PBS

|

|

|

Placed on 10 ul drop of 1/250 PBS diluted rabbit anti-cholera toxin IgG

37^{o} C for one hour then washed in PBS

|

|

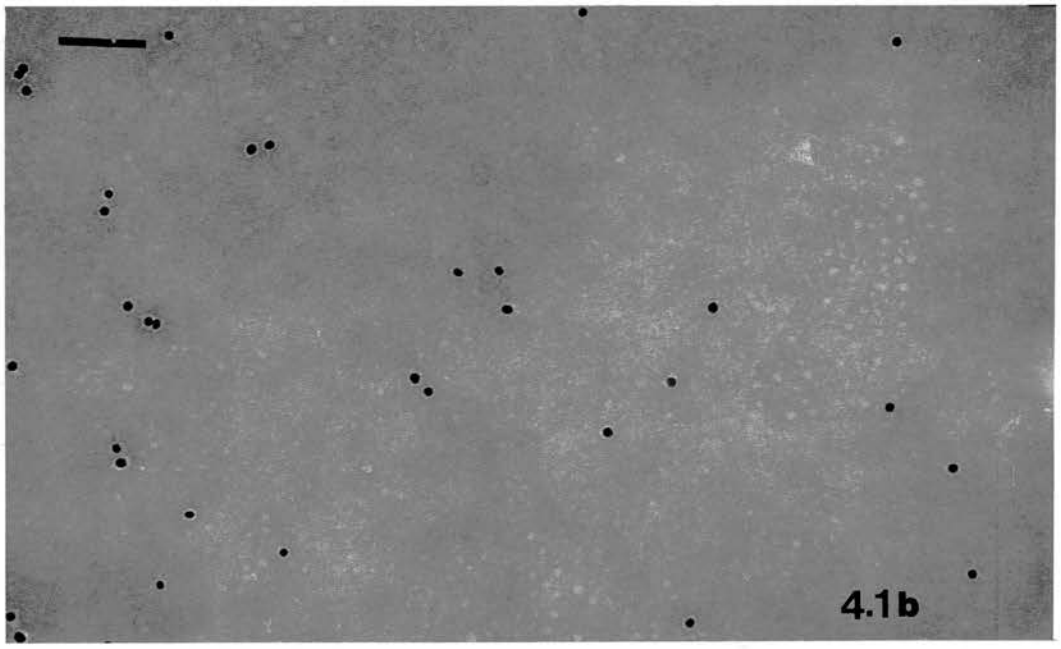
Placed on 10 ul drop of 1/50 PBS diluted gold labelled goat anti-rabbit antibody at 37^{o} C for 30 minutes then washed with PBS and water followed by staining for electron microscopy

Many control binding experiments were also performed to indicate the specificity of the reagents used. The following table summarises these controls and what they would show if binding was observed;

CONTROL PERFORMED	COMMENTS
Cholera toxin step was omitted.	This experiment indicates any non-specific binding of the rabbit anti-cholera toxin antibody.
The cholera toxin (850 ng/ml) was incubated with excess GM_1. (1 mg/ml).	This experiment shows any impurity present in cholera toxin preparation. which might bind to section.
The rabbit antiserum 1/250 PBS dilution was preabsorbed with an equal volume of 850 ng/ml cholera toxin.	This experiment also shows any non-specific binding of the rabbit anti-toxin antibody.
The rabbit anti-toxin antibody was omitted.	This experiment shows non specific binding of the goat anti-rabbit antibody to toxin or section.
Both cholera toxin and rabbit antibody step were omitted.	This experiment also shows non specific binding of the second antibody to the section.

Fig.4.1b Colloidal gold-cholera toxin (15nm) showing the 'halo' of protein (cholera toxin) adsorbed to it. The gold-cholera toxin probe was made according to the method of Geoghegan and Ackerman, (1977) and dried onto a collodion coated nickel grid before counterstaining lightly with 2% uranyl acetate.

Bar indicates 1um



The B subunit alone was used for binding (1ug/ml).

This experiment confirms that the toxin is binding to GM_1 ganglioside receptor.

4.3.0 Results

4.3.1 Binding Properties of Colloidal Gold-Toxin Probe

4.3.1.1 Examination by electron microscopy

Cholera toxin was successfully adsorbed onto the cholera toxin colloidal gold. Fig 4.2 shows a non electron dense halo surrounding the 15nm gold particles which is a layer of cholera toxin molecules adsorbed onto the gold.

4.3.1.2 Results and Discussion

The colloidal gold-toxin probe bound specifically to the plasma membrane of NIH 3T3 cells.

Figs.4.2a and b show the typical labelling pattern obtained on cells fixed and then labelled at room temperature. The labelling occurs along the plasma membrane. This labelling is specific as an excess of unlabelled toxin prevents labelling. Figs.4.2c and d show NIH 3T3 cells labelled at 37^{o}C prior to fixation. Cells labelled with the probe at this temperature internalise it into vesicles near the cell surface during the time course of 30 min before fixation.

Control cells labelled with an excess of unlabelled cholera toxin before fixation prior to labelling with the gold toxin probe are not shown as they showed no labelling with colloidal gold toxin.

The labelling on the cells fixed and then labelled at room temperature was all on the plasma membrane with no observable internalisation of the label. Because the control cells showed no labelling this indicates that the toxin adsorbed to gold binds to the same sites as unlabelled toxin. At 37^{o}C the colloidal gold-cholera toxin conjugate appeared to have labelled the cell surface more heavily and become associated with non-coated cell surface invaginations. Gold particles were also located in vesicular structures within the cytoplasm near the cell surface.

Internalisation of colloidal gold-cholera toxin conjugates has previously been commented upon by Montesano et al. (1982), in cultured liver cells. This experiment indicated that both cholera toxin and tetanus toxin gold conjugates bound specifically and entered the liver cells by flask-shaped non-coated invaginations. It was suggested that these invaginations might be a route specific for glycolipid internalisation. I essentially repeated this experiment with NIH 3T3 cells, using it to check that the cholera toxin retained its binding properties once adsorbed to gold and to see whether I could repeat the observations in a different cell line .
The cholera toxin conjugate in my experiment was generally not associated with coated pit structures but with smaller uncoated vesicles. This result is similar to that obtained by Montesano et al. (1982). These vesicles have been described by Rohlich and Allison (1976) who stated that they were only observed in some areas of the membrane and that they had a longitudinal row pattern which was partially disrupted by Cytochalasin B, indicating the involvement of microfilaments in this arrangement.

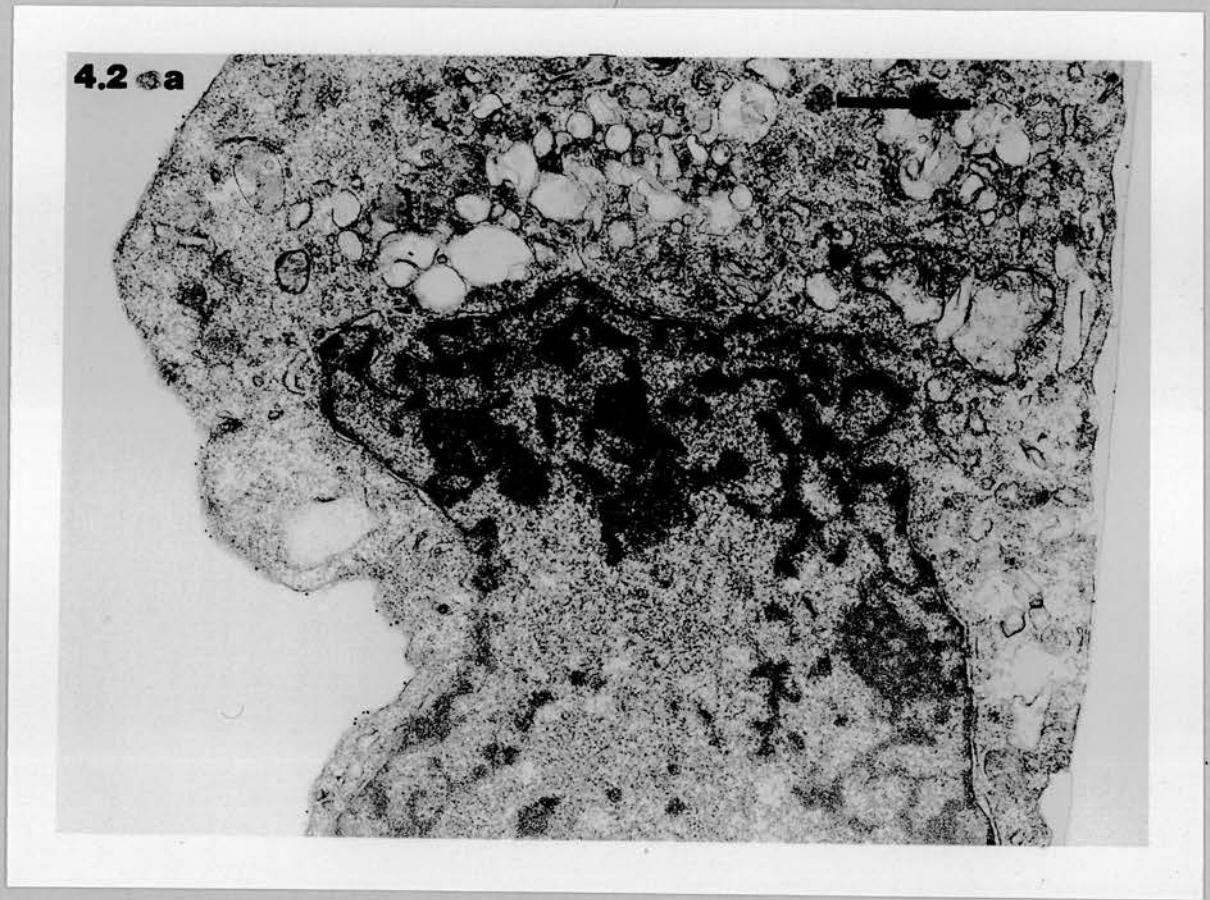
It is possible that such uncoated vesicles participate in endocytosis of macromolecules from the cell surface. The 'longitudinal row' pattern of these vesicles was not visible in the sections examined, but this may be due to angles of cutting sections failing to reveal inner vesicles.

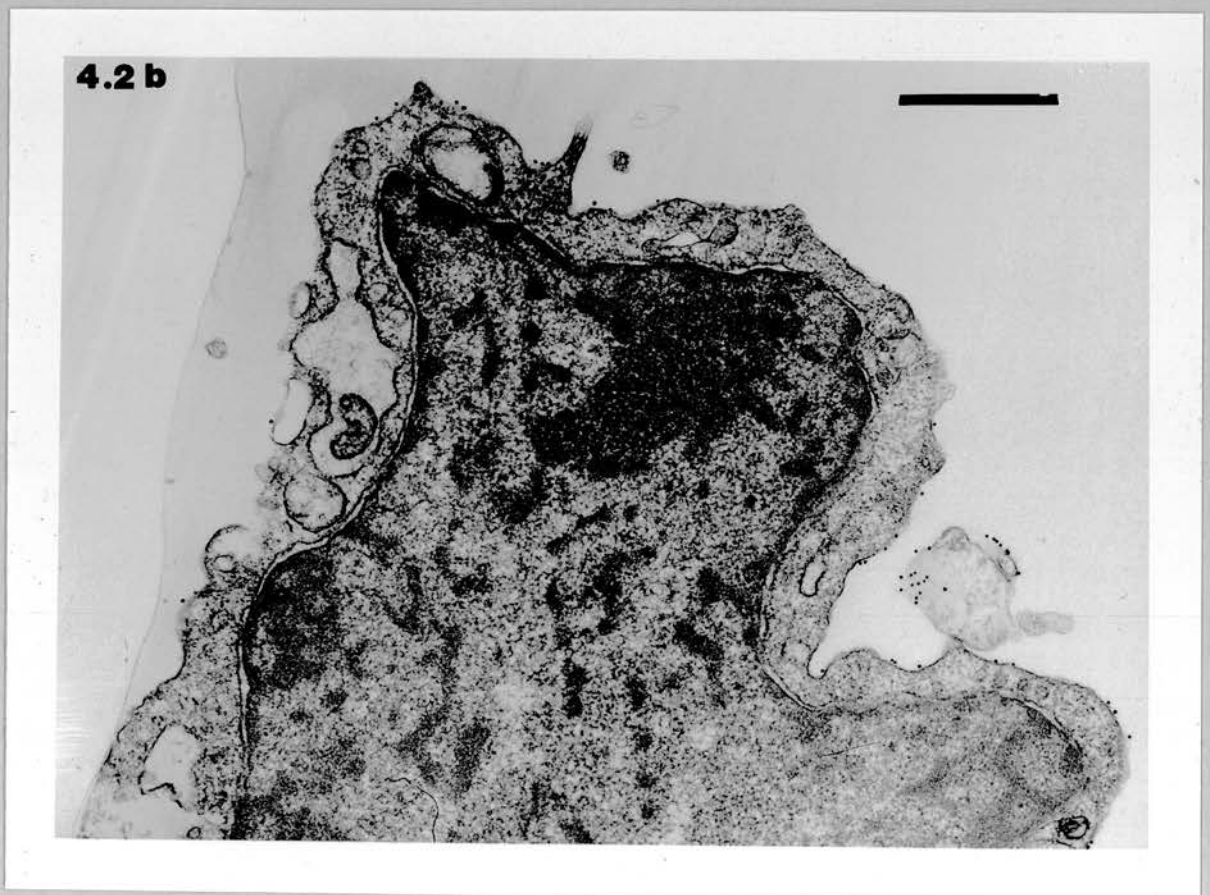
The internalisation of the toxin-gold complex via these uncoated vesicles must be a fairly slow process in NIH 3T3 fibroblasts as the much of the complex was still present on the cell surface. Joseph et al. (1979) found that peroxidase labelled cholera toxin was taken up and reached the golgi of murine neuroblastoma cells within thirty minutes. Using rat liver and ^{125}I-labelled toxin, Janicot and Desbuquois (1987) showed that the whole toxin was taken up after fifteen minutes and was associated with endosomes, lysosomes and the golgi during this time. All these experiments confirm that the GM_1 receptor is probably recycled but it is not clear whether this process requires the presence of the toxin as in all cases it is presumed that the toxin was bound to the receptor during internalisation

These experiments therefore indicate that there is a mechanism for uptake of whole toxin in cells which bind the toxin at their surface. NIH 3T3 cells and liver cells both bound the gold toxin probe specifically at their surface and were able to internalise this probe. The experiments also show that the GM_1 receptor may be internalised into the cell with cholera toxin bound to it. Internalisation was observed by virtue of the bound toxin probe.

Figs.4.2a,b NIH 3T3 cells incubated with colloidal-gold cholera toxin at room temperature for 30 min. Note that the plasma membrane shows a sparse localisation of gold particles.

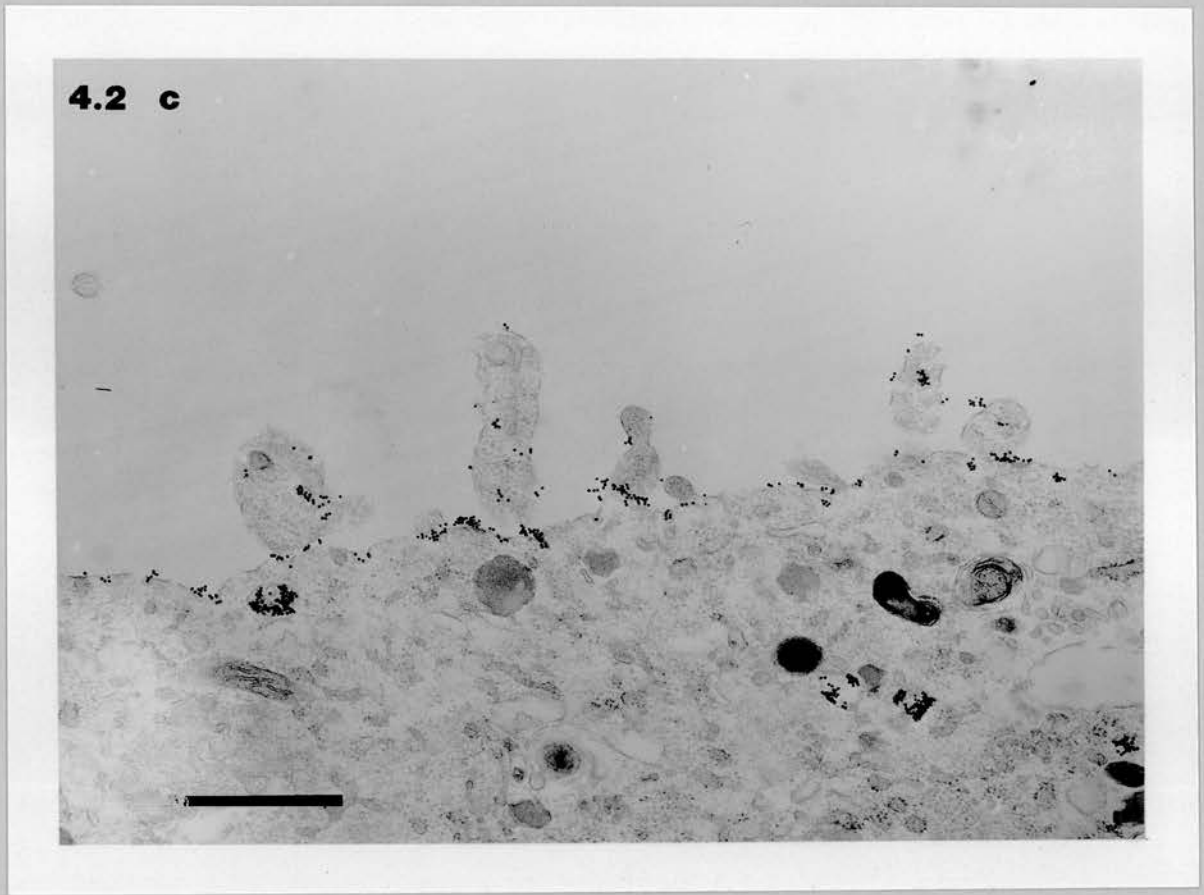
Bar indicates 1um

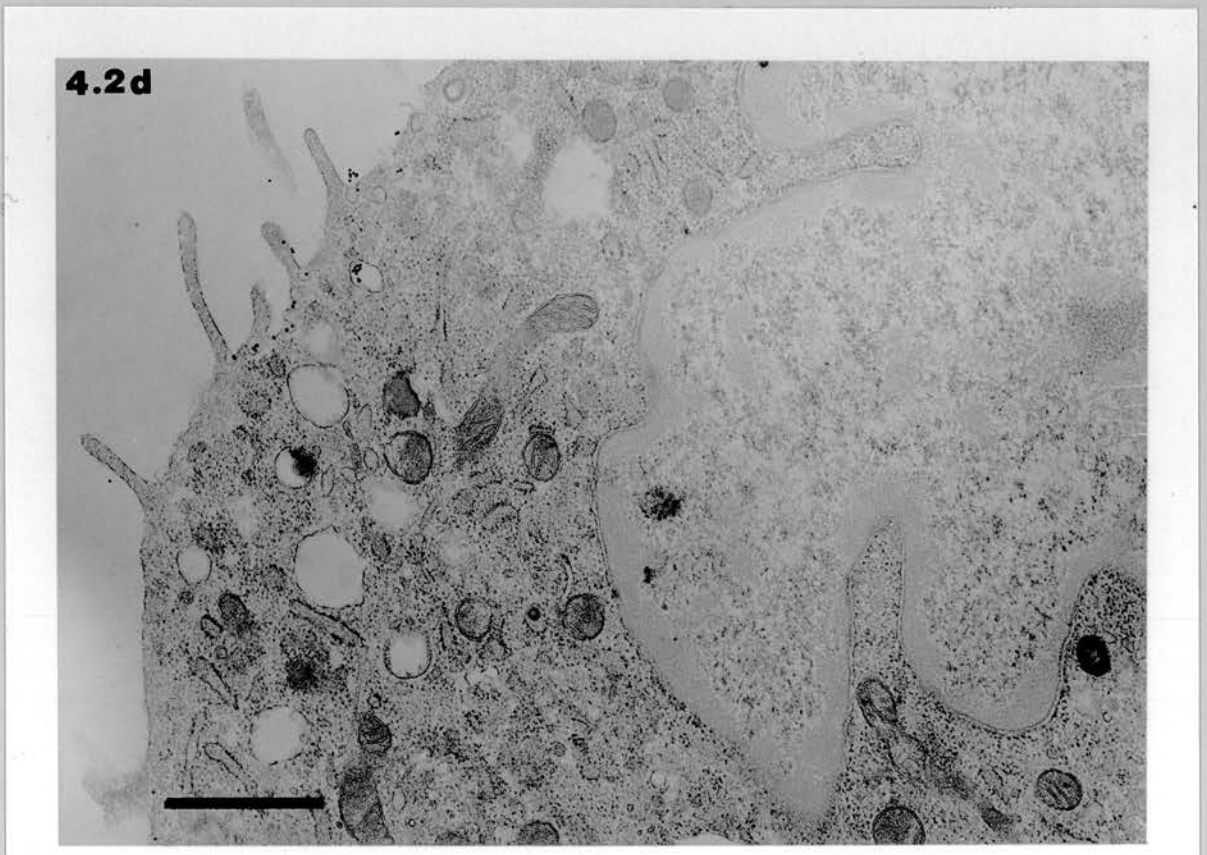

4.2 a


4.2 b

Figs.4.2c,d NIH 3T3 cells incubated with colloidal gold cholera toxin at 37°C for 30 min. Gold particles are found in non-coated invaginations on the plasma membrane and in lysosomes

Bar = 1um


4.2 c


4.2d

4.3.2 Localisation of Cholera Toxin Binding Sites on Sections of Mouse Small Intestine

4.3.2.1 Binding of the colloidal gold-toxin probe to mouse small intestine

None of the dilutions of colloidal gold toxin probe that were tested (1/50 to 1/500) bound to the sections of mouse small intestine. One explanation for this observation might be that the gold conjugate was too large and sterically hindered its own binding because of the proximity of the binding sites on the tissue section. This hypothesis could have been verified by preparing additional gold toxin probes with gold of varying diameters but there was insufficient time to prepare several batches of different sized gold and the cost of toxin prohibited this analysis. Another reason might have been that the protein was no longer attached to the gold or had reduced binding properties. These reasons also could have been tested by repeating the NIH 3T3 binding experiment, but loss of protein was thought to be unlikely. The most plausible reason may have been due to reduced hydrophilicity of the resin (personal communication Dr Arthur Rowe, Dept. of Biochemistry, University of Leceister). I decided therefore to try and label the mouse small intestinal sections with toxin itself followed by a sandwich of antibodies. This approach did produce labelling and indicates possibly that the toxin probe could not bind strongly enough to the resin due to its lack of hydrophilicity

4.3.2.2 Results of binding using cholera toxin followed by antibodies

The results presented in this section have been reproduced at least 10 times on both mouse and rat tissue.

Cholera toxin bound sparsely to the microvilli, to sites within vesicular structures in the cytoplasm and, most surprisingly, both whole toxin and B subunit alone bound to the heterochromatin of the nuclei (see fig.4.3b and c). This binding was specific, as all the controls (except one) to test specificity of the reagents produced no gold labelling on the section.

CONTROL PERFORMED	COMMENTS
Cholera toxin step was omitted.	Some binding was observed only to organism outside intestinal tissue. No binding on intestinal tissue. See fig. 4.3e.
The cholera toxin (850 ng/ml was incubated with excess GM_1 (1 mg/ml).	No binding was observed
The rabbit antiserum 1/250 PBS dilution was preabsorbed with an equal volume of 850 ng/ml cholera toxin.	No binding was observed. See fig. 4.3f.

CONTROL PERFORMED	COMMENTS
The rabbit anti-toxin antibody was omitted.	No binding was observed. See fig 4.3d.
Both cholera toxin and rabbit antibody step were omitted.	No binding was observed

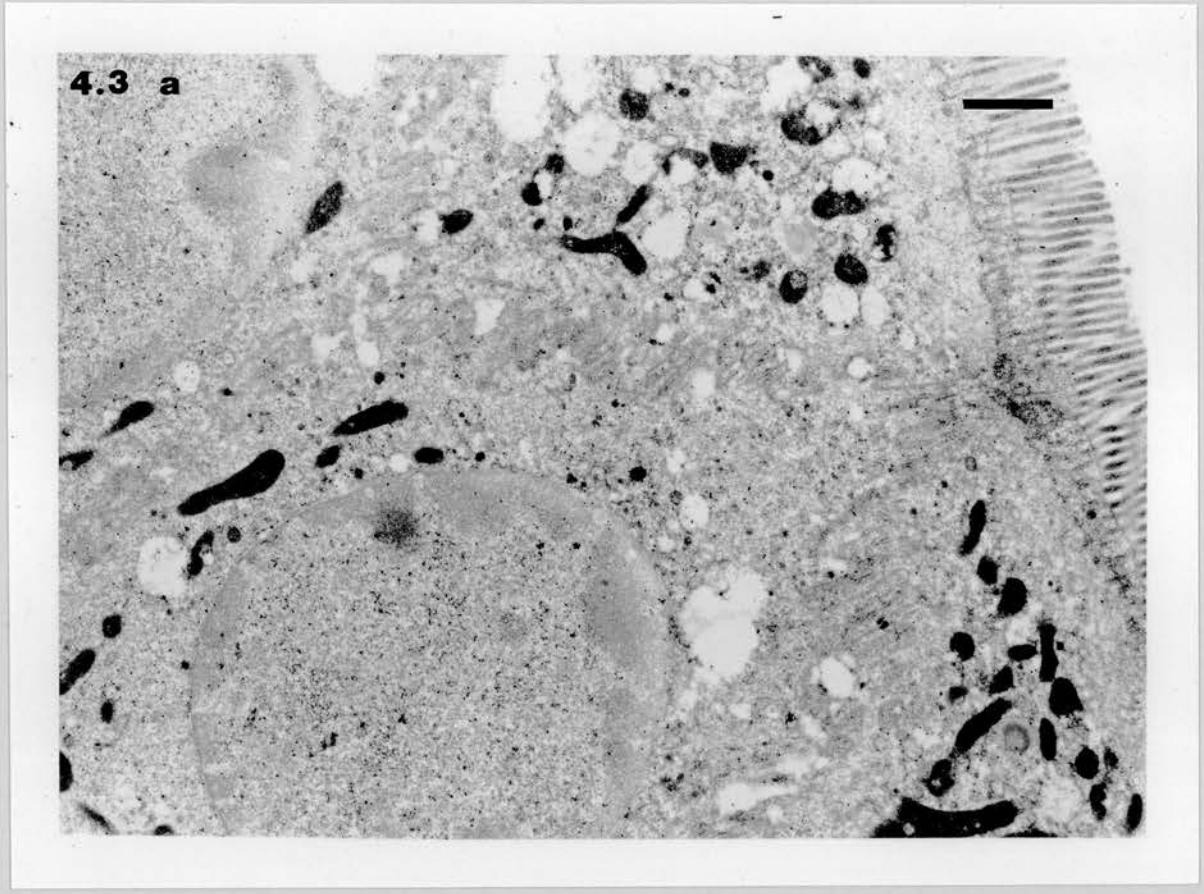
On the labelled control the gold particles were observed on an organism attached to the microvilli.
It was presumed that this organism was the E.coli strain which produces a toxin very homologous to cholera toxin. The gold particles were only observed around the organism and not on the gut tissue itself (see fig.4.3e).

Anti-GM_1 antibody also bound both to the nucleus and the microvilli (see fig.4.3a) indicating the presence of GM_1 by an independent ligand. Some of these sites might not be ganglioside GM_1 as this antiserum did show some cross reactivity with asialo ganglioside GM_1 and some reactivity with ganglioside GD_{1b} (personal communication from Dr.N.Gregson). This binding pattern was also observed in rat intestine.

Fig.4.3a Shows the binding of anti ganglioside GM_1 antibody (1/50 dilution in PBS) to a section of mouse intestine

Fig.4.3b Shows the novel heterochromatin binding site of the B subunit (1ug/ml) of cholera toxin to a section of mouse intestine

Bar = 1um


4.3 a

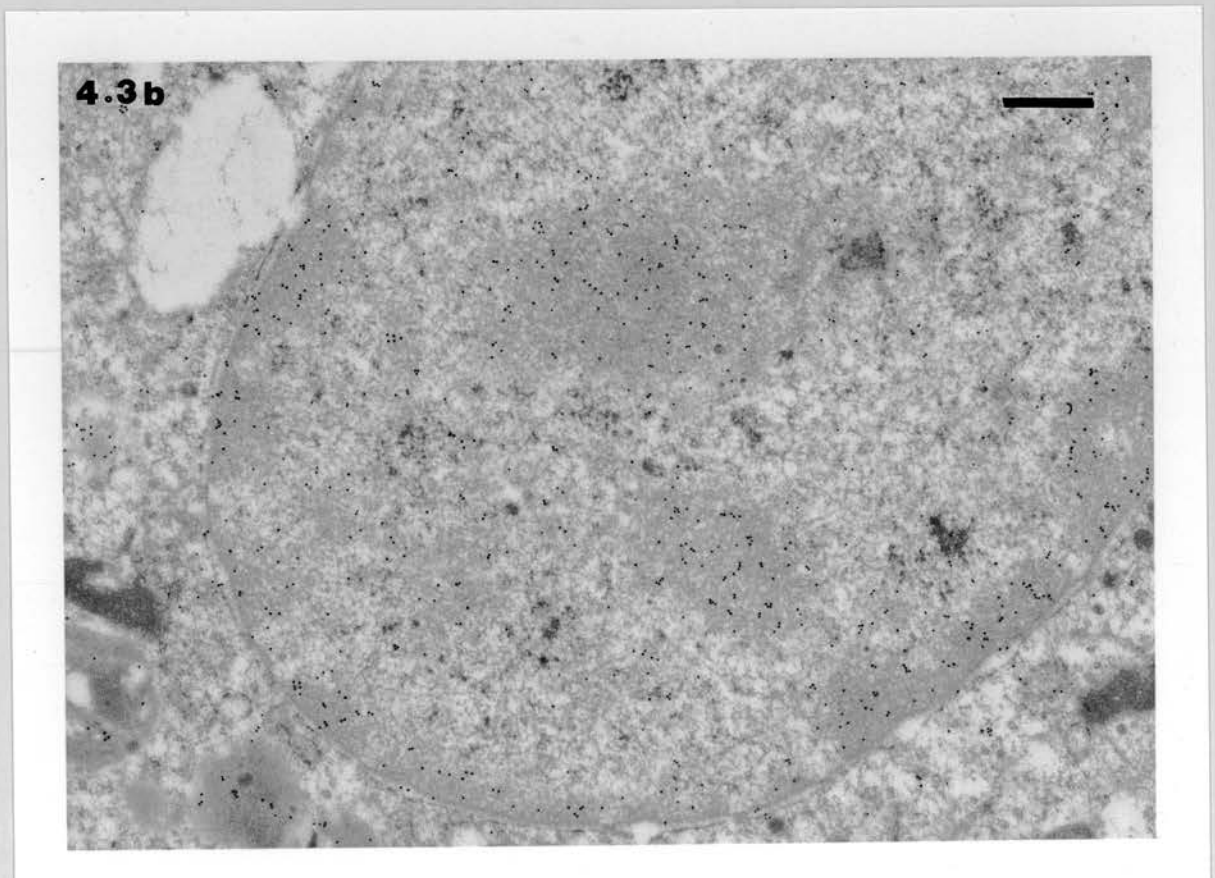
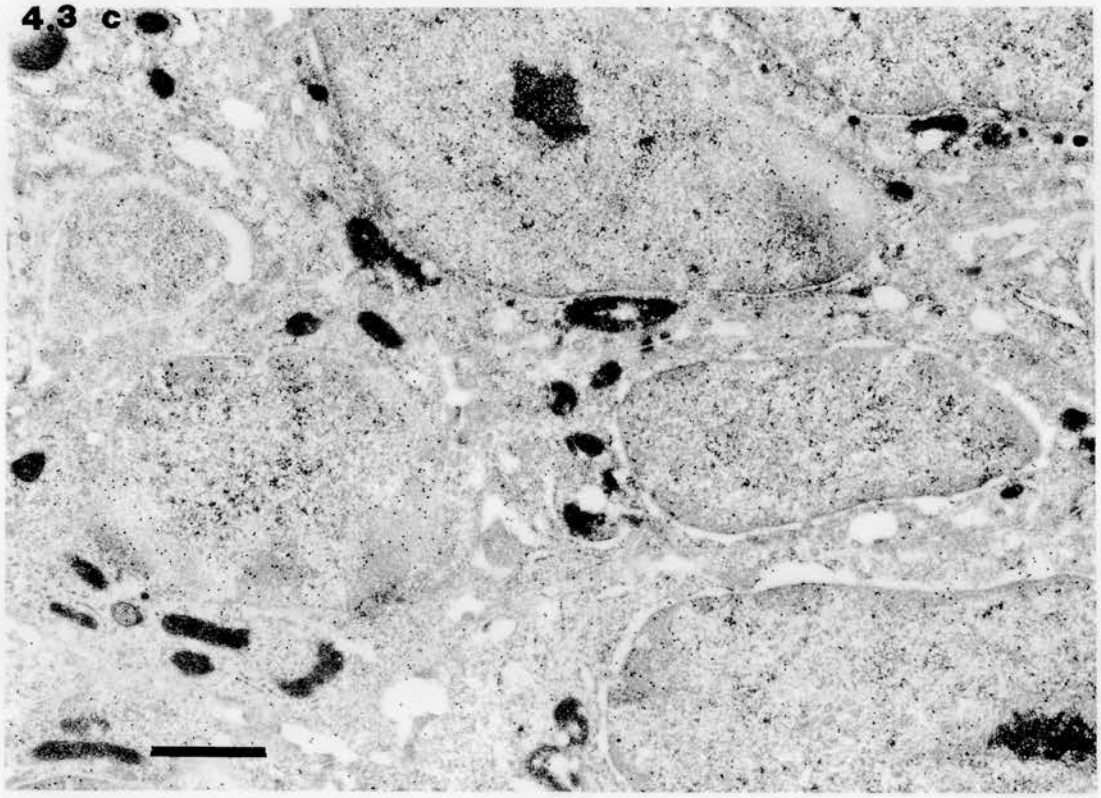

4.3b

Fig.4.3c Binding of whole cholera toxin (850ng/ml) to a section of mouse intestine

Fig.4.3d Control (primary antibody omitted)

Bar = 1um


4.3 c

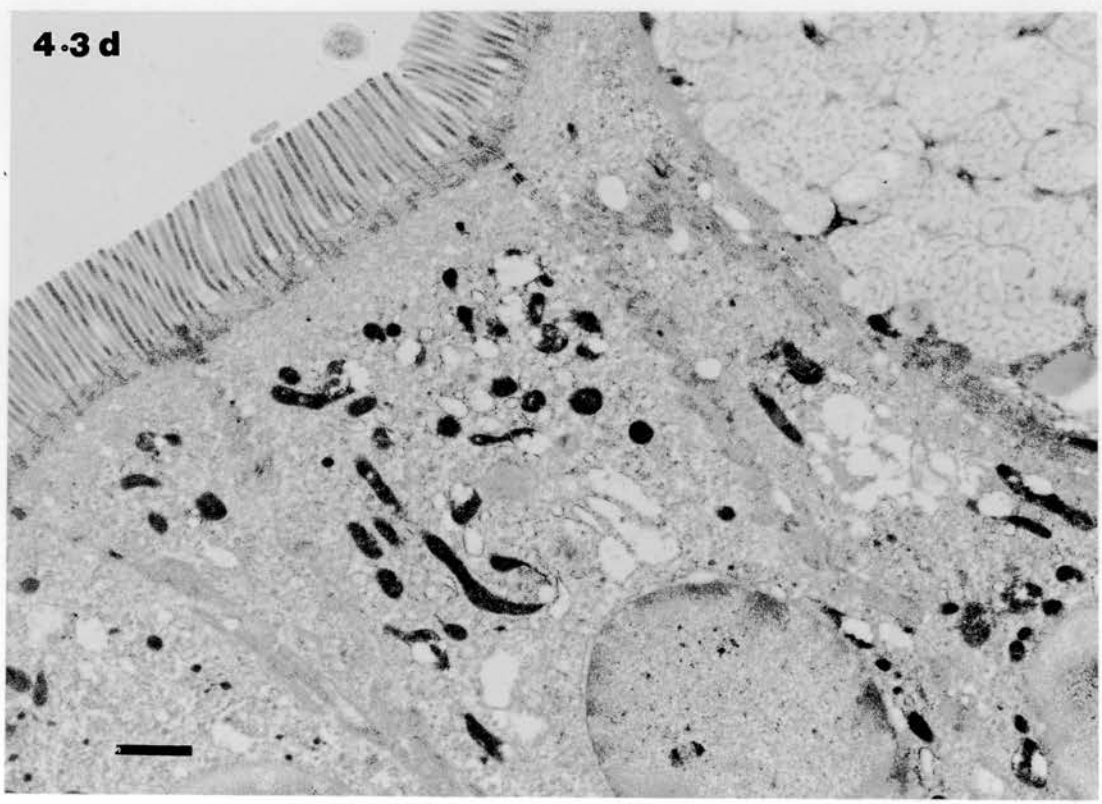
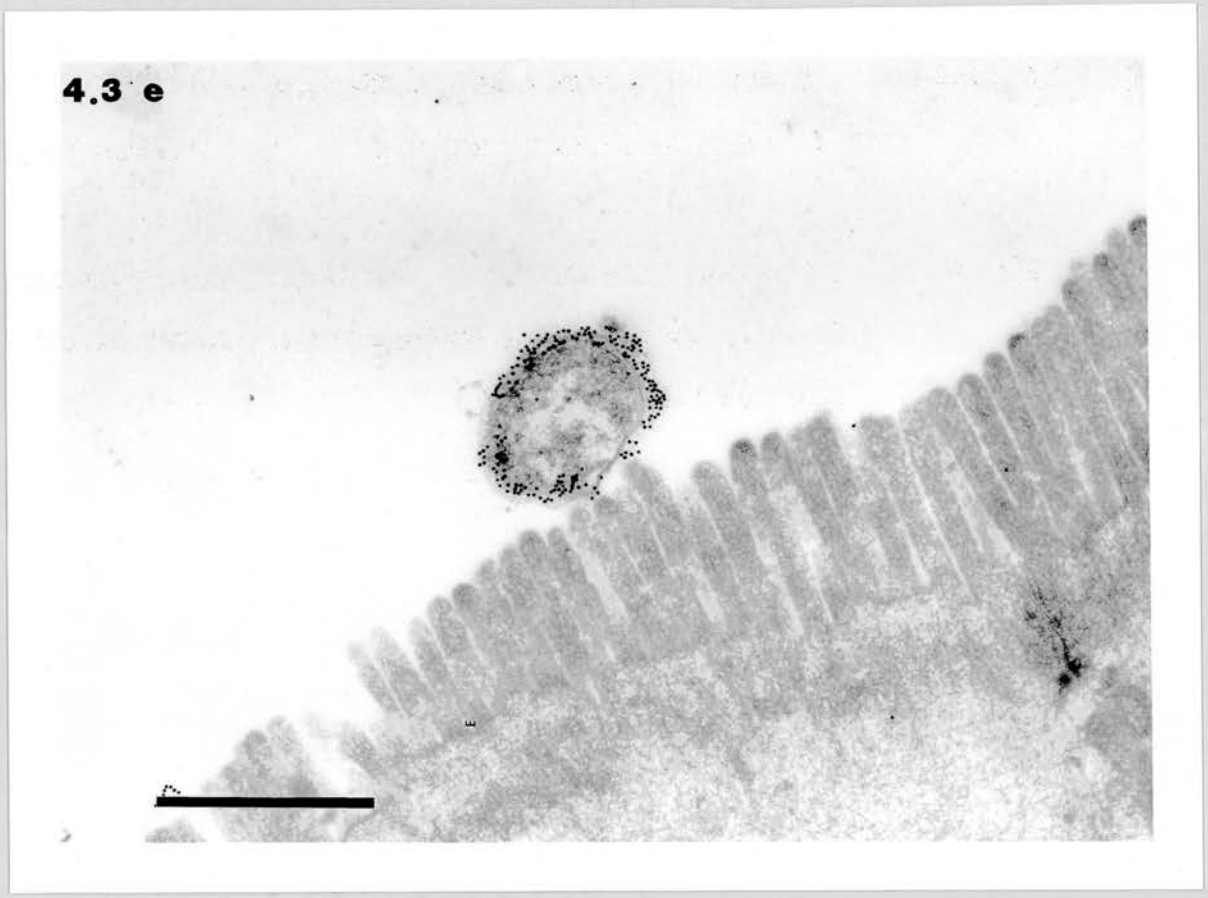
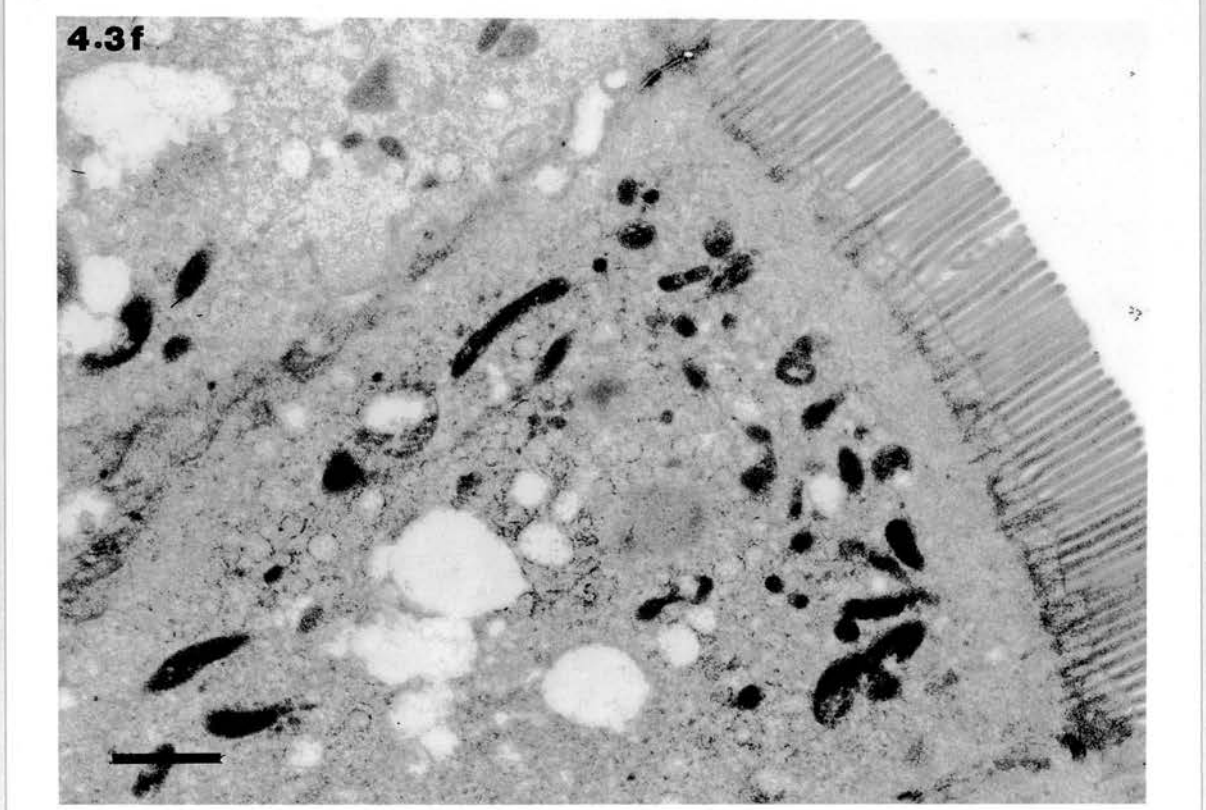

4.3 d

Fig.4.3e Control (cholera toxin omitted) indicating the binding of the primary antibody to a gut organism, probably E.coli.

Fig.4.3f Control: pre-incubation of cholera toxin with primary antiserum (850ng/ml Cholera toxin + 1/250 dilution primary antiserum in PBS v/v)

Bar = 1um


4.3 e


4.3f

4.4.0 Discussion

In this experiment I decided to exploit the high binding affinity of cholera toxin for GM_1 ganglioside and the sensitive post-embedding immunogold localisation technique to probe for extracellular and intracellular cholera toxin binding sites which I presumed would represent the GM_1 distribution of the cell. I used mouse small intestinal tissue as this is a target tissue for cholera toxin.

In previous immunocytochemical experiments to locate GM_1 ganglioside on cell surfaces, cholera toxin was the GM_1 probe because of its known specificity for the molecule.

One of the early ganglioside GM_1 localisation experiments using the electron microscope was performed by Hansson in 1977. The cells Hansson examined were incubated with cholera toxin at room temperature ($18^{o}C$+/- 1) and then rinsed. Following this, the cells were immersed in peroxidase conjugated anti-cholera toxin immunoglobulin for 30 mins and then fixed and further stained. A large number of cells (200-1000) were examined. Binding was also carried out at $4^{o}C$ and $37^{o}C$ and controls were studied too where the toxin had been omitted before tissue processing or with omission of the antibody step. Hansson showed that binding occurred predominantly on the plasma membranes and his results correlated well with previous workers who had estimated the levels of GM_1 on different cell membranes. Hansson also examined the binding of cholera toxin on the clinical target - human small intestine. He observed that the microvilli showed patchy labelling. There was a smaller amount of precipitate on lateral and basal membranes. A prominent binding region was the caveolar membranes and the junctional complexes between adjacent epithelial cells (Hansson et al., 1977).

The colloidal gold technique has also been applied to the localisation of GM_1 on human blood cells and bone marrow (Ackerman et al., 1980). In these experiments IgG-$F(ab')_2$ anti-cholera toxin was used to prevent non-specific activity with F_c receptor sites on monocytes. The Fab fragments were adsorbed to the colloidal gold and used to localise cholera toxin which had previously been incubated with cells or marrow.

Binding of cholera toxin to intestinal cells to locate intracellular GM_1 sites by post-embedding immunogold has been examined once previously. In this case gold-toxin probe was used which bound to the cell specifically (excess unlabelled toxin prevented its binding). Labelling was found on the plasma membrane and intracellularly in vesicles in addition to the golgi apparatus but there is no mention of any nuclear binding. The main differences between this experiment and my own was the use of human rather than mouse intestinal tissue and the use of different fixative and embedding procedures to process the tissue (Roth, 1985). The difference in results may well therefore reflect a difference in intracellular gangliosides between mice and human tissue or provide evidence that some fixation/embedding procedures may abolish or prevent detection of some cellular components.

In addition to locating GM_1 externally and internally on cells by immunocytochemical means, workers have also tried to locate the molecule intracellularly by biochemical fractionation techniques (Keenan et al., 1972 a, b). These experiments indicated that perhaps only a small number of gangliosides were located on the plasma membrane compared to the total cell content of gangliosides.

4.4.1 Are the binding sites GM_1?

The results presented show potential binding sites for cholera toxin associated with heterochromatin in the nucleus. It seems likely that these sites are ganglioside GM_1, the only receptor for the toxin that has been positively identified. This conclusion is supported by the observation that preincubation of the toxin with ganglioside GM_1 inhibits the binding, presumably because the binding site (on the toxin molecule) is no longer available (although conceivably because of transmitted conformational change to a different binding site). Further support comes from the fact that anti GM_1 antibody binds to sites parallel to those of toxin in the same tissue. It is true that GM_1 antibody binding did not interfere with that of toxin but this could be explained if, as is probable, the affinity of the antibody for GM_1 is lower than that of toxin, or if anti-GM_1 antibody does not bind to all possible sites. From previous cell fractionation experiments, it is known that ganglioside in mammary and liver tissue is mainly associated with plasma membranes, but some was found in other cellular membranes and it was not a result of cross contamination from the plasma membrane (Keenan et al., 1972 a, b). However, the heterochromatin localisation of ganglioside has never been suggested. Current evidence suggests that ganglioside synthesis begins in endoplasmic reticulum, then there is transport to the golgi and termination at the plasma membrane (Keenan et al., 1974; Lipsky and Pagano, 1985). At no stage is the nucleus thought to be involved. It is possible that a conformational epitope is being recognised in the nucleus by the antibody and not an actual glycolipid. This phenomenon has already been reported by Freddo et al. (1986) who showed that a monoclonal anti-DNA antibody bound to a conformational epitope formed by phosphatidic acid and gangliosides.

Interferons which bind to a ganglioside receptor and modulate growth (like the B subunit of cholera toxin) (Spiegel, 1988) have intranuclear binding sites (Burwen and Jones, 1987).

The question of intranuclear receptors for growth factors and polypeptide hormones was critically discussed by Evans and Bergeron (1988) who argue that the evidence for such intranuclear sites is equivocal (further discussed in Chapter 5).

It is also possible that the binding site observed could be a glycoprotein with a similar structure. Cell surface glycoproteins having immunochemical properties similar to GM_1 ganglioside have been identified in fibroblasts (Tonegawa and Hakamori, 1977). Additionally nucleoplasmic glycoproteins are known to exist (Hart et al, 1988).

Despite the lack of additional data to support ganglioside GM_1 localisation in the nucleus, it still remains true that cholera toxin is probably the most sensitive available probe for GM_1.

4.4.2 Relevance of a possible GM_1 binding site

These binding results do not imply that the nuclear binding sites can be reached by the toxin when it is working in the intact cell or *in vivo*, nor do they in themselves suggest any physiological role for binding in the nucleus. However these results do beg the question "What is the role of ganglioside GM_1 in the heterochromatin of the nucleus?"

Experiments on internalisation of peroxidase-conjugated or ^{125}I- labelled toxin into intact cells have shown the toxin to be associated with a number of intracellular organelles eg. the golgi complex (Joseph et al., 1979). There has been no evidence for any particular binding to the nucleus.

There is evidence, however, that gangliosides are associated with cell growth and differentiation and so perhaps with gene expression in the nucleus.

Altered ganglioside metabolism and organisation have been related to oncogenic transformation, cell cycle and density dependent growth inhibition (Fishman and Brady 1976; Hakamori,1981; Feizi, 1985). Several studies have also indicated that the crosslinking (by antibody or pentavalent cholera toxin) of cell surface gangliosides either endogenously derived or exogenously supplied, can regulate DNA synthesis (Spiegel and Fishman, 1987). Polyanionic micelles of GM_1 ganglioside have also been shown to inhibit DNA synthesis in isolated nuclei by affecting the activity of DNA polymerase alpha fractionated from S-Phase Hela cells (Ohsawa et al., 1988).
Therefore there is implication of ganglioside involvement at a nuclear level but no direct proof exists yet.

CHAPTER 5

BIOCHEMICAL EVIDENCE FOR A CHOLERA TOXIN BINDING SITE IN INTESTINAL CELL NUCLEI

CHAPTER 5

BIOCHEMICAL EVIDENCE FOR A CHOLERA TOXIN BINDING SITE IN INTESTINAL CELL NUCLEI

5.1.0 INTRODUCTION

The work described in Chapter 4, using the technique of immunogold electron microscopy, indicated the existence of a novel binding site for cholera toxin in mouse intestinal cells. The toxin was shown to bind to the dense chromatin, primarily located near the nuclear membrane of mouse small intestinal cells.

This specific binding of cholera toxin is intriguing, as it points to the presence of a GM_1-like molecule within the nucleus. The binding site might be physiologically relevant to the action of cholera toxin. Preparation of the tissue for examination by electron microscopy involves many chemical reagents which might have produced an artificial binding site although the controls performed do indicate the presence of a GM_1 ganglioside-like molecule. It was decided therefore, to attempt to demonstrate this toxin binding site by biochemical methods. The site was probed for by Western blotting of nuclear membrane fractions which would still have associated chromatin, but this yielded no evidence of binding. Simple direct binding experiments were also performed with radioiodinated cholera toxin. These latter experiments did indicate the presence of a specific binding site.

5.2.0 Preparation of Nuclear Envelope Membranes from Rabbit Intestinal Cells

5.2.1 Isolation of Intestinal Epithelial Cell Nuclei

An adult New Zealand white rabbit was sacrificed by cervical dislocation and its small intestine removed and placed in a 0.9% w/v sodium chloride solution (saline).

The gut contents were forced out, by washing through with the saline solution. The gut was then cut into 10cm lengths, and these tubes were cut along the mesenteric border. The mucosal cells were scraped gently using a glass slide and placed in 50mM mannitol buffer containing 4mM magnesium chloride and 5mM Hepes buffer pH 7.5. The cells were homogenized in a Jencons Potter-Elvehjem homogeniser. The mixture was then centrifuged at 800g for 10 min ($4^{o}C$) in a Beckman J2-21 centrifuge in a Ja-14 rotor, to remove cell debris. The supernatant was removed and the pellet was resuspended in the same buffer. The resuspended mixture was centrifuged at 1000g for 10 min ($4^{o}C$) in the same rotor to pellet the nuclei.

The pellet was taken up in 70% sucrose solution to make an effective sucrose solution of just under 56%. This was then layered onto 56% sucrose and spun at 60 000g for an hour at $4^{o}C$ in a 50Ti rotor in a Beckman L8-55 ultracentrifuge. This process separates nuclei from contaminating small cell debris. Finally, the pellet was washed with the 50mM mannitol buffer to remove the sucrose (Bloebel and Potter, 1966). This preparation of nuclei was then further processed.

5.2.2 Nuclear Membrane Preparation

Dr. Paul Agutter from Napier College, Edinburgh has been studying the nuclear membranes for several years and has written several books on the subject (Agutter, 1986).
He is therefore experienced in the preparation of nuclear envelope membranes and associated chromatin. He advised me to use the following adapted technique of Harris and Milne (1974) to prepare the nuclear envelope membranes with associated heterochromatin.

The nuclei were resuspended in a 1mM $NaHCO_3$ solution (pH 7.2-8.0) until the absorbance of the solution at 400nm read between 0.1-0.2 units of absorbance (method communicated by Dr. Agutter to give a suitable dilution of nuclei).

The mixture was equilibrated for five minutes before centrifugation at 20 000g for 10 minutes at 4°C in a JA-20 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended in the bicarbonate solution which had been warmed to 25°C and the mixture was left for fifteen minutes. Then DNAase 1 was added to a concentration of 10ug/ml. The solution was centrifuged again as before at 20 000g and again the pellet was resuspended.

A cycle of resuspension and centrifugations followed until the A 260nm absorbance reading (absorbance of nucleic acid) was reduced to less than 0.5 units of absorbance. The A 260nm readings gave an indication of the amount of DNA still in the preparation.

The final preparation of nuclear envelope and associated chromatin was run on a 12% SDS PAGE gel (see section 2.2.5 and fig 5.0) and compared with published preparations (Kaufmann et al., 1983), which were found to be similar.

In addition, because there were no antibodies available to verify the presence of nuclear membrane proteins, the SDS PAGE gels were examined by Dr. Agutter who confirmed the protein pattern to be characteristic of nuclear membrane and associated proteins (personal communication Dr. P.S. Agutter).

This preparation was stored at -20^{o}C in 1ml aliquots with protein concentrations of 0.06mg/ml and 2mg/ml.

5.3.0 Immunoblotting of Nuclear Envelope Preparations Transferred onto Nitrocellulose

Nuclear envelope preparations were loaded onto 12% SDS PAGE gels (about 70mg protein) and electrophoresed and then transferred onto nitrocellulose as described in section 2.2.12.

Two of the tracks of the nitrocellulose containing molecular weight markers and nuclear envelope and associated proteins respectively were stained with Amido Black (see section 2.2.12). The other half of the nitrocellulose was soaked in a solution of 1% BSA and 2% Tween overnight to block non-specific protein binding sites on the nitrocellulose. The blot was then cut into strips representing individual tracks of protein. Each strip was then subjected to a different immunostaining protocol (see Table 5.0).

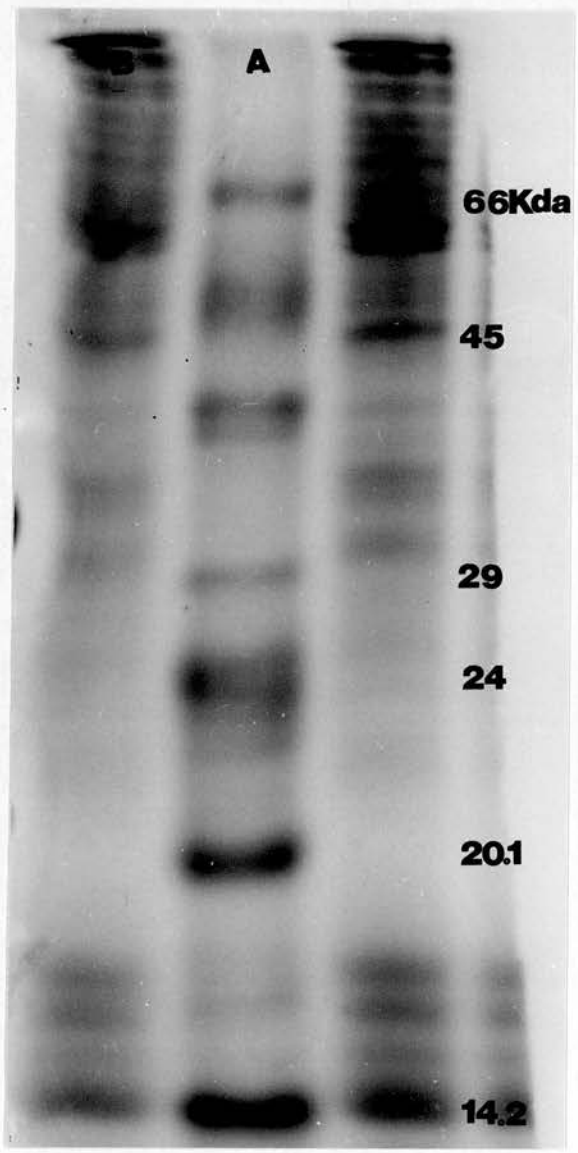
5.3.1 Results

Cholera toxin failed to bind to any component of the nuclear envelope preparations after the proteins had been separated by SDS PAGE on a 12% gel and blotted onto nitrocellulose.
In order to test the procedure a positive control was carried out. Cholera toxin (50ug) was run on a 12% SDS PAGE gel and blotted onto nitrocellulose.
There was apparent binding at 100ug/ml of cholera toxin but this was shown to be non-specific. Strips incubated with cholera toxin at concentrations of 10 and 50ug/ml did not indicate any binding.

This was then immunostained with a 1/250 dilution in PBS of the primary antibody and a 1/100 dilution also in PBS of the peroxidase conjugated second antibody obtained from the Scottish Antibody Production Unit (SAPU). The B subunit of cholera toxin showed staining using this procedure, indicating that the experimental protocol was working.

The possible reasons for the observed lack of cholera toxin binding are discussed in section 5.7.2.

FIG 5.0 12% SDS PAGE Gel Showing the Proteins Extracted From the Nuclear envelope Extraction Procedure of Harris and Milne (1976).



A = Molecular weight markers

B = Nuclear envelope and associated proteins

TABLE 5.0 Immunostaining Protocol for Nitrocellulose Strips of Nuclear Envelope Protein.

Treatment					
	1	2	3	4	5
30 min at 37°C in 5ml of	10µg/ml CT	50µg/ml CT	100µg/ml CT	100µg/ml CT	PBS 1% BSA 2% TWEEN
	3 x 5 ml washes in PBS 1% BSA 2% TWEEN				
60 min at 37°C in 5ml of	1/250 rabbit anti-(cholera toxin) antiserum (raCTa)			PBS 1% BSA 2% TWEEN	1/250 (raCTa)
	3 x 5 ml washes in PBS 1% BSA 2% TWEEN				
60 min at 37°C in 5ml of	1/100 HRP labelled goat anti(rabbit IgG) antiserum				
	3 x 5 ml washes in PBS 1% BSA 2% TWEEN				
Stained in	1 vol 3mg/ml 4-chloro-1-napthol in methanol + 3 vol PBS + hydrogen peroxide(3µl/10ml buffer)				
	extensive washes in distilled water				

5.4.0 Binding of ^{125}I-labelled Cholera Toxin to Membranes

5.4.0.1 Direct Binding Assay

As the immunoblotting procedure failed to show cholera toxin binding, a direct binding assay was done on the membranes as there was a strong likelihood that the strongly denaturing conditions of the SDS PAGE or absorption to the nitrocellulose blot had abolished the binding site or that the binding site was just not available under these conditions.

A simple binding assay was carried out, similar to that performed by Griffiths et al. (1986), to look at the properties of cholera toxin binding sites on rabbit intestinal brush border membranes and the nuclear envelope membranes.

Nuclear envelope membranes were diluted in 55mM Tris-HCl buffer containing (80mM NaCl 10mM mannose 3mM K_2HPO_4 1mM MgCl 1mM $CaCl_2$ pH 7.4) (Tris-buffer) to give a protein concentration from 10 to 650ug/ml.

Then 250ul of membrane suspension were aliquoted into 1ml Eppendorf tubes which had previously been incubated with a 1% BSA solution to reduce adsorption of radioactive toxin to the plastic tubes. The membranes were then incubated with 50ul of diluted ^{125}I-labelled cholera toxin (see section 2.2.6) in the same buffer as the membranes to give an effective toxin concentration of between 0.05-5.00nM. All test samples were done in duplicate. Control tubes had at least a 40 fold excess of unlabelled toxin also. The tubes were then incubated in a 37^{o}C water bath for one hour. Following this, the tubes were centrifuged at 13 000g for 10 min in a microfuge at room temperature and then the supernatant was removed.

The pellets were then washed twice by resuspension and centrifugation at 13 000g (microfuge) in the Tris buffer. Finally, the radioactivity of the pellets was counted in a gamma counter (LKB 1282). Brush-border membranes from the same tissue (prepared and donated by David Longbottom), Longbottom and van Heyningen, (1989) were also included in the assay as these membranes possess known cholera toxin binding sites (ganglioside GM_1).

5.4.0.2 Binding Assay to Plastic Microtitre Plates

As the membrane binding assay used a large amount of membranes and expensive amounts of cholera toxin, a microbinding assay was done which meant binding could be done in triplicate and all reagents used in much smaller volumes. Nuclear envelope membranes (0-50ul) of a 0.7mg/ml solution were pipetted into the wells of a microtitre plate (Falcon Microtest III) and the volume was made up to 50ul with 1mM bicarbonate buffer pH 7.6. The wells were covered and left overnight at $4^{o}C$. The following day, the protein was removed and the wells were washed three times with PBS containing 1% BSA and 0.05% Tween 20 (blocking buffer). The wells were then filled with the blocking buffer and incubated for 2 hr at room temperature. Then 50ul of an 18ug/ml ^{125}I-labelled cholera toxin solution (prepared as in section 2.2.6) was added to the wells then incubated at room temperature for one hour. Controls included pre-incubating the wells for 30 min with 1mg/ml unlabelled cholera toxin, or incubating the radioactive cholera toxin solution (18ug/ml) with 2mg of GM_1 ganglioside for 30 minutes prior to labelling the wells.

5.5.0 Adenylate Cyclase Assay and Marker Enzyme Assay

The nuclear envelope membranes were prepared twice by the same method (Bloebel et al., 1966). As apparently specific cholera toxin binding was observed with both preparations, it was necessary to show that there was no significant plasma membrane contamination which might have produced a false positive result. Therefore each of the preparations were tested for one of two enzymes, thought to be specific for the plasma membrane. The first preparation was subjected to an adenylate cyclase assay using the method adapted from White (1974). The second preparation was tested using the sucrase marker enzyme assay of Dahlqvist (1968). Both these assays were kindly performed for me by a colleague, David Longbottom of Edinburgh University. The results are presented in sections 5.6.2 and 5.6.3 respectively.

5.6.0 Results

5.6.1 Direct Binding Data

Initial experiments with the first batch of nuclear envelope membrane were repeated several times. The results presented are representative experiments showing the mean of the duplicates (which did not differ more that 10%), with non-specific binding allowed for by subtracting the values obtained for the control which was always about 5% of the total counts added (see Fig.5.1.a,b). The binding of cholera toxin appeared to reach saturation at 6nM when the nuclear membrane protein concentration was about 400ug/ml (see Fig. 5.1.c). In the other binding experiments shown the membrane protein concentration (nuclear envelope) was also at 400ug/ml or greater. It is apparent from the other binding curves that saturation was not reached (Figs. 5.1a,b).

Toxin concentrations above 5nM could not be used for all the experiments performed as the cost of the toxin was prohibitive. An interesting point to note is that the amount of toxin bound per microgram of protein is much greater for the nuclear envelope preparation than for the brush-border preparation. This result indicates that even if the nuclear envelope preparation actually consisted of the same amount of plasma membrane protein, this would not be enough to explain the extra binding capacity.

5.6.1.2 A Scatchard analysis of Binding

Toxin binding is thought to be multivalent which is likely as there are five binding subunits (Fishman and Atikaan, 1979) and the binding is essentially irreversible ($K_d = 10^{-9}M$) to plasma membranes of cells possessing the GM_1 receptor, both factors which have to be considered if one makes an analysis of the binding by a Scatchard plot. The analysis assumes that all potential binding sites are equivalent which may not be true for cholera toxin which can bind to up to five GM_1 ganglioside molecules, and the probability of binding could change once the toxin is already partially bound. The actual GM_1 molecules (or other binding sites) are not necessarily the same either.

Despite the limitations of this analysis it was employed as a useful tool to get one analysis of the type of binding that was observed with cholera toxin on nuclear envelope preparations. A Scatchard analysis was performed on the binding data from Fig.5.1b (Scatchard, 1949). The Scatchard plot is presented in Fig. 5.2. The Scatchard plot is clearly non-linear with a host of possible interpretations, one being an indication of negative cooperativity, possibly representing two different binding sites with different affinities for the toxin.

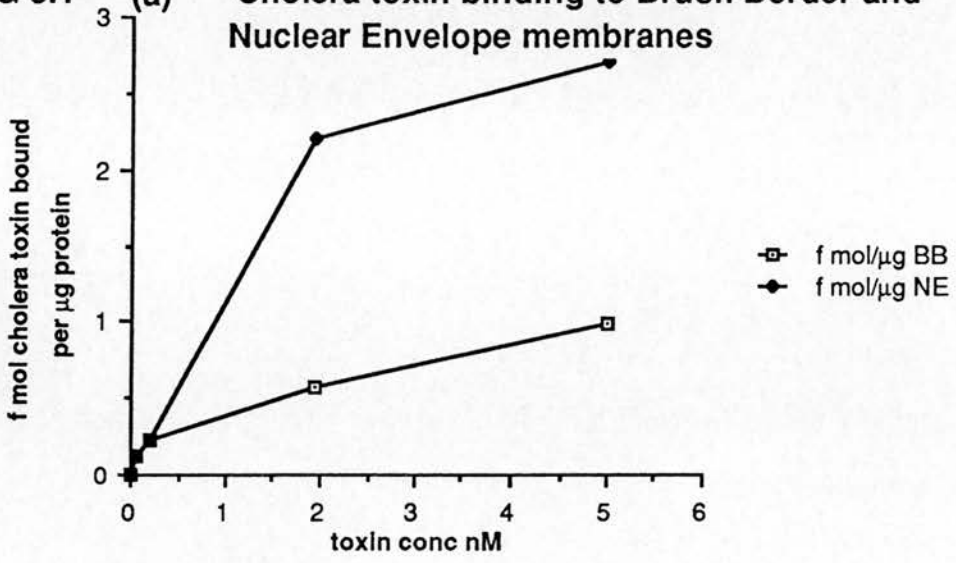
Another explanation of the non-linearity of the Scatchard plot may be that it represents a set of identical sites whose affinity decreases with increasing receptor occupancy by the ligand (Klotz, 1986). In the following analysis of the data the former binding model (two different binding sites) was assumed. Again it must be stressed that this is the simplest of interpretations of the plot. The major limitation with this assumption is that saturation was not achieved and thus the binding affinities especially the lower ones are extrapolated, a frequent occurrence in Scatchard when it is rarely possible to perform binding between 1% and 99% saturation which makes the plot more difficult to interpret (Zierler, 1989). The binding model used to examine the binding data may be represented by the following equation:

$$b = N_1.f / (K_{d1} + f) + N_2.f / (K_{d2} + f)$$

b	=	concentration of bound ligand
N_1, N_2	=	concentrations of binding sites for site 1 and 2
K_{d1}, K_{d2}	=	dissociation constants of each binding site for ligand
f	=	concentration of free toxin

The above model was fitted to the experimental data using a program for a non-linear regression written by Dr. G. Atkins of the Edinburgh Biochemistry Department. The program was written in IMP and run on the Amdahl 470 computers of the Edinburgh University Computing Service.

FIG 5.1 (a) Cholera toxin binding to Brush Border and Nuclear Envelope membranes



(b) Cholera toxin binding to nuclear envelope membranes

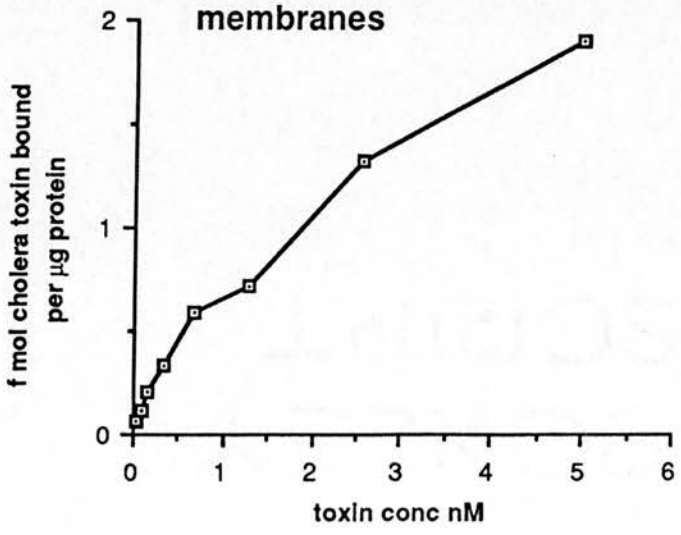


Fig 5.1 (c) The Effect of Nuclear Envelope Membrane concentration on Cholera Toxin binding

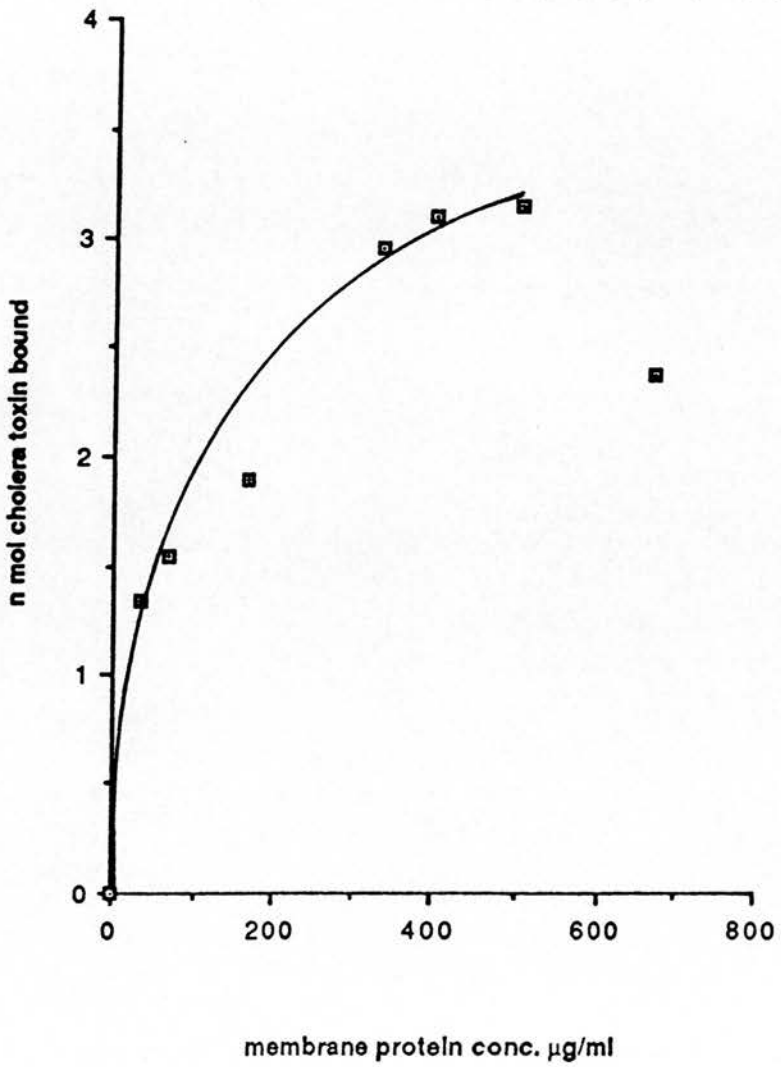
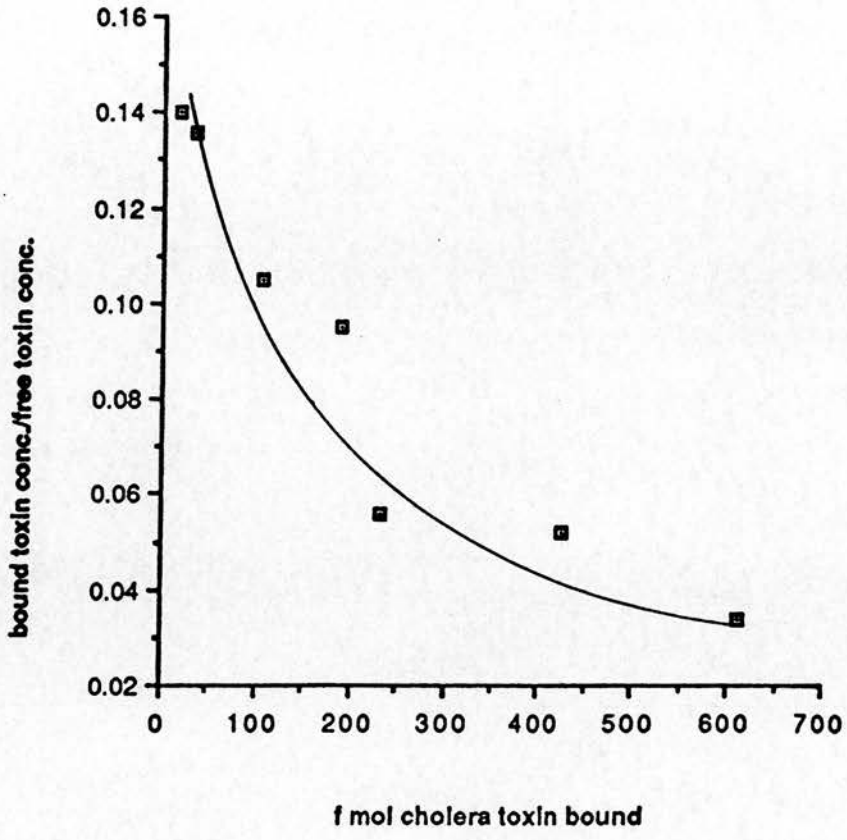


FIG 5.2 **Scatchard Plot**



INTERCEPTS ARE NOT SHOWN AS THIS INFORMATION WAS GENERATED BY COMPUTER

The least squares procedure was that of Marquardt (1963). The data were weighted equally. The Scatchard plot was useful for providing initial estimates for the non-linear regression analysis.
These estimates were:

N_1 = 0.33nM

K_{d1} = 2.00nM

N_2 = 3.30nM

K_{d2} = 70.0nM

(NB. The reaction mixture volume was 300ul and the protein concentration was 400g/ml)

The curve fitting refined these estimates to (mean and standard deviation indicated):

N_1 = 0.29nM +/- (0.42nM)

K_{d1} = 2.99nM +/- (4.24nM)

N_2 = 3.70nM +/- (1.29nM)

K_{d2} = 69.5nM +/- (66.1nM)

Therefore allowing for the volume of the binding reaction (300ul) the two theoretical K_d values may be about 2nM and 70nM respectively if indeed there were two different binding sites (Note, however, the high standard deviations on these figures). Taking the amount of cholera toxin bound, the total numbers of the two sites could be $N_1 = 4 \times 10^{12}$ sites per milligram of protein and $N_2 = 6 \times 10^{13}$ sites per milligram of protein respectively.

If the toxin was binding monovalently these numbers could represent the total number of each type of binding site present. But as stated these binding interpretations are just the simplest analysis of cholera toxin and its binding to nuclear material because an alternative analysis of one binding site which binds with increasing affinity to the toxin could equally be true.

The result from the microtitre plate binding assay is represented by Fig.5.3. This graph appears to be reaching saturation at about 55fmol toxin per well. As can be seen, preincubation of the toxin with GM_1 ganglioside abolished the binding. This experiment is discussed together with the previous results in section 5.7.1.

5.6.2 Adenylate Cyclase Assay

The value obtained for the adenylate cyclase activity in the first preparation of nuclear envelope and associated proteins was 0.056 pmol/min/mg protein). This result was measured using the method of Salamon (1979). In similar experiments with plasma membrane fractions from the same intestinal tissue, David Longbottom obtained values of 30 pmol/min/mg protein for the adenylate cyclase activity (the basal level being less than 1). The difference in activity between the nuclear preparation, and the plasma membrane preparation may indicate how little plasma membrane contamination was present within the nuclear envelope preparation.

However, adenylate cyclase is a readily inactivated enzyme. The nuclear preparation was not 'flash' frozen in liquid nitrogen but at $-20^{o}C$ where the possibility of ice crystal formation exists.

Therefore, if adenylate cyclase had been present it might have been inactivated by the freezing process.

5.6.3 Sucrase Enzyme Assay

The assay for sucrase (Dahlqvist, 1968) which is specific for plasma membranes was kindly performed by David Longbottom on both a nuclear envelope preparation (2nd preparation) and a brush-border membrane preparation from the same rabbit intestine. The membranes were kept on ice and prepared on the same day and frozen in liquid nitrogen at the same time. The results suggest that about a thirtieth of the nuclear preparation is due to contamination from the brush-border preparation (see table 5.1).

These results indicate very clearly the lack of contamination from the brush-border membranes which contain known cholera toxin binding sites.

5.7.0 Discussion

5.7.1 Binding Data

These preliminary binding results strongly support the evidence for a specific cholera toxin binding site in the nucleus.

The lack of saturation obtained may reflect a large number of lower affinity sites present which is consistent with the Scatchard analysis (6 x 10^{13} sites per milligram of protein). The lack of saturation might also represent internalisation of the toxin into nuclear membrane vesicles.

As the nuclear envelope preparation was not examined by electron microscopy it is not known whether vesicles of membrane were actually present, but Harris and Milne (1974) state that the nuclear envelope preparation method does produce some intact nuclear ghosts. The binding assay was performed at 37^{0}C for one hour (a temperature at which internalisation could take place).

Previous workers looking at cholera toxin binding in brush-border membranes carried out the incubation at 0^{o}C (Griffiths et al., 1986) or in chicken intestinal cells, at 37^{o}C (Hyun and Kimmich, 1984) and on HeLa cells at 4^{o}C and 37^{o}C (Fishman and Atikaan, 1980).

Hyun and Kimmich (1984) stated that in their binding assay only 4% of ^{125}I -labelled toxin binding could be reversed after 15 minutes incubation at 37^{o}C and that this represented the percentage of internalised toxin into cells. They also analysed the stability of the toxin receptors by pre-incubating the cells at 37^{o}C before adding ^{125} I-labelled toxin to determine whether the nature of the binding changed.

In my experiment I was dealing with nuclear membrane fragments and associated heterochromatin. The possibility exists that nuclear membrane vesicles were also present and that the lack of saturation represents toxin being internalised into these vesicles. I did not determine the binding at any other temperature, because I wanted to reproduce as much as possible the binding conditions used in the electron microscopical binding experiments (see section 4.4.2).

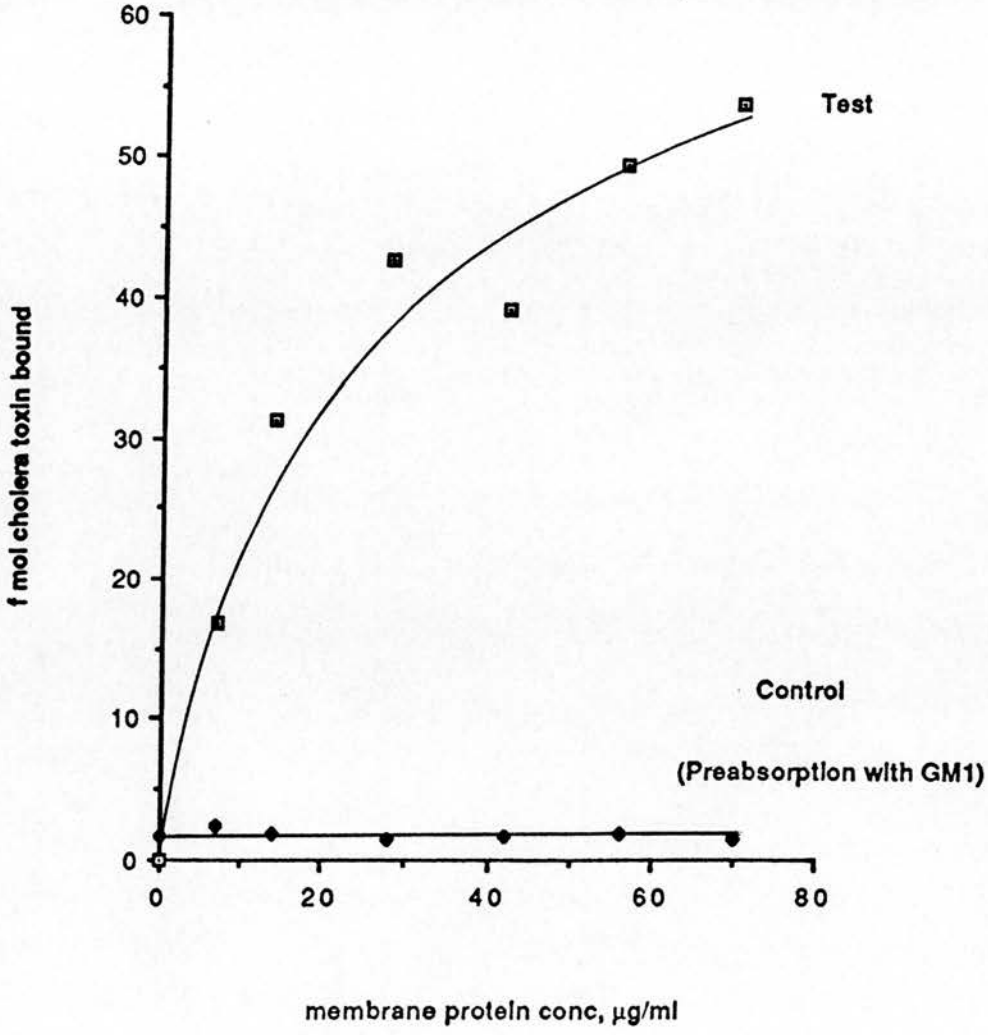
The K_d values of about 2nM for the possible high affinity site is the same order of magnitude as previously calculated K_d values for cholera toxin binding to GM_1 ganglioside (Cuatrecasas, 1973a; Donta et al., 1982; Griffiths et al., 1986).

The possibility that GM_1 exists in the nucleus is discussed in section 5.7.3. The binding of toxin to membrane was prevented by preincubation with GM_1 ganglioside which further indicated that the binding site might be GM_1 ganglioside (see Fig.5.3).

The adenylate cyclase assay performed on the first nuclear preparation suggests that these membranes did not contain plasma membrane contamination. As the adenylate cyclase enzyme might have been destroyed by the freezing process in the preparation of the membranes the lack of contamination is not conclusive. Therefore the second preparation was strictly temperature regulated and 'flash' frozen in liquid nitrogen to maintain any potential enzyme activity.

The sucrase assay performed on this preparation clearly indicated that only 3% of the nuclear envelope preparation was contamination by brush-border membranes (see table 5.1). The binding site therefore was present in the nuclear membranes.

FIG 5.3 Microtitre plate binding assay for cholera toxin to nuclear envelope membranes



5.7.2 Immunoblotting Data

The lack of binding indicated by the blotting technique (Section 5.3.1) does not negate the immunocytochemical and direct binding assay results both indicating the presence of a specific nuclear binding site for cholera toxin.

There are many reasons why this technique did not show up a binding site. Some of these are shown below:

1) The binding site may have been reduced by the mercaptoethanol treatment, so altering its conformation.

2) The site may be bound to the nitrocellulose and thus not available for cholera toxin binding.

3) The SDS may have irreversibly denatured the binding conformation of the site.

4) The binding site, if lipid (ie. GM_1) might have been dispersed by the detergent.

TABLE 5.1 SUCRASE ASSAY RESULTS

	PROTEIN CONC.	SUCRASE ACTIVITY			
MEMBRANE	**μg/ml**	**U/ml**	**U/mg**	**U**	**PF**
H	3.56	0.369	0.104	14	1.00
MPM	2.07	0.425	0.205	36.4	2.00
BBM	1.49	1.230	0.826	26.3	8.00
N	0.0625	0.0016	0.026	0.01	0.25

H= CRUDE CELL HOMOGENATE

MPM= MIXED PLASMA MEMBRANES

BBM= BRUSH BORDER MEMBRANE PREPARATION

N= NUCLEAR ENVELOPE MEMBRANE PREPARATION

U=UNITS OF SUCRASE ACTIVITY

U/mg=SPECIFIC ACTIVITY

PF=PURIFICATION FACTOR COPARED TO CRUDE CELL HOMOGENATE

5.7.3 Ganglioside Location Within the Nucleus

This subject was dealt with in chapter 4 but a few more points are made here. The cellular location of gangliosides was mentioned in section 1.2.2.2. In an extensive review Franke (1974), indicated that no glycolipids could be found in the nucleus in biochemical extractions performed up till then.

Prior to the publication of this review, Keenan et al.(1972b), had detected gangliosides within the nucleus, but judged this material to be contamination, as it showed the same protein content as plasma membrane. Later, Dnistran et al. (1979) also showed gangliosides to be present within rat liver nuclei. They observed 1.00 nmol of glysophingolipid per mg of protein in their nuclear fraction. This corresponds to about 6 x 10^{15} potential binding sites per mg of protein which is within two orders of magnitude of one of my calculated numbers of cholera toxin binding sites. However, this calculation assumes all the glycophingolipid sites to be GM_1 ganglioside, which they are not (Dnistran et al., 1979).

More recently Matyas and Morre (1987) also detected gangliosides within the nucleus of rat liver. They stated that 5% of total cell gangliosides were detected within nuclei. Therefore, this evidence together with the cholera toxin binding evidence presented in this study indicates the existence of GM_1 ganglioside within nuclei.

The possibility exists that there is a glycoprotein with a similar carbohydrate composition to ganglioside GM_1. Both N-acetyl galactosamine and N-acetyl glucosamine linked glycoproteins have been found within the nucleus (Gerace et al., 1982; Snow et al., 1987).

5.7.4 Physiological Relevance of Cholera Binding Site in the Nucleus

Previous work on cholera toxin internalisation into cells has not shown cholera toxin to localise within the nucleus (Joseph et al., 1979; Janicot and Desbuquois, 1987). However, Joseph et al. (1979) used the peroxidase staining of cholera toxin which is difficult to observe within cells and Janicot and Desbuquois (1987), detected ^{125}I-labelled toxin which had internalised after cell fractionation of liver, which was not a very precise method to localise cholera toxin.
However, as cholera toxin was not expected to go to the nucleus it has never been specifically looked for in this organelle.

In section 1.1.2 it was shown that some of the effects of cholera toxin in cells cannot be correlated with increases in adenylate cyclase activity. Recently Spiegel and Fishman (1987) looked at the effect of the B subunit binding of cholera toxin to ganglioside GM_1 and found that it both stimulated DNA synthesis and cell division in quiescent, non-transformed mouse 3T3 cells and inhibited the growth of *ras* transformed 3T3 cells. They explain this result in terms of the role of GM_1 ganglioside and not B subunit of cholera toxin.
They offer no mechanism by which the B subunit could modulate the growth response of cells both positively and negatively. It could be that binding of the B subunit to ganglioside GM_1 affects the interaction of the ganglioside with nuclear components.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.0 Introduction

In this chapter the work presented in this thesis will be briefly discussed with regard to the current theory of cholera toxin action on cells and what is known about GM_1 ganglioside. In addition some further experiments are suggested which might be performed to further address those aspects of the toxin action and GM_1 ganglioside function and location which remain to be elucidated.

6.1 Cholera Toxin

6.1.1 Cholera Toxin Binding and Internalisation

As described in chapter one section 1.1.2 there are three basic steps to intoxication by the cholera toxin protein and these are:

1. Binding of the toxin to the cell surface.
2. Internalisation of part or all of the toxin molecule.
3. Activation of the adenylate cyclase enzyme.

With regard to binding, there is overwhelming evidence that the toxin binds predominantly to the GM_1 ganglioside. It is known that the toxin molecule is able to bind up to five GM_1 ganglioside molecules as it has five B subunits each able to bind to one GM_1 ganglioside molecule. However, it is not clear whether binding to one or more gangliosides is a prerequisite for its further action.

The next step is internalisation of the active A_1 subunit of the toxin from the extracellular surface towards the cytoplasmic surface where the G protein of the adenylate cyclase enzyme complex is located. It is this area which remains unclarified. It is known that binding of whole toxin to GM_1 ganglioside induces a conformational change in the toxin molecule as measured by shift in the wavelength of tryptophan residues. Additionally, toxin binding to lipid bilayers containing GM_1 ganglioside perturbs the lipid bilayer inducing pore formation. Furthermore, there is a lag time of 15-60 minutes between cholera toxin binding and onset of adenylate cyclase activity. The variability in the lag time is due to different incubation conditions (there is a temperature dependency) and different cell types. The suggestion is that the lag time reflects a membrane translocation of A_1 subunit and at least four different mechanisms have been proposed which are presented in chapter one, section 1.1.2.2. The final event is the permanent activation of the adenylate cyclase enzyme.

In chapter 3 of this thesis the first measurements of the lateral mobility of bound cholera toxin and additional lateral mobility measurements of its cellular receptor GM_1 are presented. The receptor had been inserted into the membrane of NIH 3T3 mouse fibroblasts which had their normal complement of ganglioside GM_1 in the membrane. The mobility of the bound fluorescent toxin and of the inserted fluorescent ganglioside receptor were similar with a lateral diffusion coefficient of $1x10^{-9}cm^2s^{-1}$ and an immobile fraction of 20%. These results indicate that lateral mobility is insensitive to size of the mobile complex and that the bound toxin does not affect ganglioside mobility in this cell line and that within the constraints of this type of physical measurement there was no evidence of any other membrane phenomenon occurring after toxin binding ie measurable patching which would have been

indicated by increasing immobility of molecules with time. These experiments also indicate that cholera toxin does not appear to cause extensive crosslinking of GM_1 receptor with immobile cell components. No other conclusions may be drawn from these measurements of lateral mobility.

There was uncertainty in the lateral mobility experiments performed as to the binding pattern of the cholera toxin molecule. If a monomeric AB toxin could be made, this could then be tested on the cell system for its affect on lateral mobility of toxin and receptor or other combinations of A and B. The A subunit could be linked to the B subunit by an easily cleaved attachment eg. a disulphide bond. It is known that cellular mechanisms exist to cleave the disulphide bond linking the active A_1 peptide from the A_2 peptide, these might also act on the bond between the A and B subunit. One could also perform the experiment linking the subunits by a non cleavable bond, but such experiment might address more the question of how the active component of the toxin reaches its site of action. Internalisation of the toxin was not an area that was examined in this thesis. In chapter 4 it was shown that the NIH 3T3 mouse fibroblasts used in the lateral diffusion studies were able to internalise a gold-labelled toxin probe but this experiment was used merely to show that cholera toxin adsorbed to gold did not lose its ability to bind to cells and be internalised.

Despite the fact that cholera toxin has been studied fairly intensively over the last twenty years, as mentioned previously the precise mechanism by which the A_1 peptide reaches its site of action (the GTP binding protein of adenylate cyclase) remains to be elucidated (see section 1.1.2.1).

It is clear there is a mechanism which exists in cells to internalise whole toxin (sections 1.1.2.1; 4.3.1.2). There is a lack of experimental data linking observations of internalisation of whole toxin or A_1 subunit alone with activation of adenylate cyclase. The major problem is one of sensitivity (ie. detecting one cholera toxin molecule with activity) and a continuous assay of the effect of toxin on a single cell needs to be worked out. Such an experiment might employ the sensitive fluorescent indicators of intracellular Ca^{2+} ions, (Fura 1 and Fura 2). There could be a correlation between the number of cholera toxin molecules bound and the increase in intracellular calcium concentration. Dixon et al. (1987) showed that the growth stimulatory effects on rat lymphocytes of the B subunit alone are mediated by an increase in $[Ca^{2+}]$; resulting from a net influx of extracellular calcium. This cellular model could then be probed with the sensitive post-embedding immunogold technique to localise whole toxin and individual subunits too. The combination of two such experiments would provide an insight in the numbers of molecule which bind to and/or get internalised into a cell but it would then have to be linked to a further experiment in the same cell on levels of toxin required for activation of adenylate cyclase.

Both sets of experiment which determine a relationship between toxin binding and internalisation and toxin binding and adenylate cyclase activation would have to be performed under strictly the same conditions of temperature and media to allow a link to be made between the amount of toxin binding and amount of adenylate cyclase activation in the cell. Such experiments would have been performed had they been easy, but immunogold localisation of internalised molecules is still difficult to perform in itself and it may take a long time to optimise conditions of localisation within cells.

Adenylate cyclase activity studies are also very difficult to perform consistently. It may be however that there is no specific mechanism for internalisation and cholera toxin gets to its site of action by being attached to GM_1 ganglioside and exploiting its internalisation route.

6.1.2 Cholera Toxin Novel Binding Site/Intracellular Localisation of GM_1 Ganglioside

Experiments performed in this study suggest the possible nuclear location of GM_1 ganglioside (section 4.3.2.1; publication at end of thesis). This binding site must be GM_1 or a very similar molecule as all the controls performed point strongly toward this interpretation of the binding of the toxin.

One of the other possible interpretations of this observation of a nuclear binding site could be that a nuclear glycoprotein with a very similar carbohydrate moiety to GM_1 ganglioside is located in the heterochromatin of the nucleus. There is no evidence to show that cholera toxin does reach the nucleus of cells on which it acts but the above finding should be further examined to see whether toxin does affect any nuclear functions and the nucleus should also be further examined and analysed for GM_1 ganglioside.

The function of the unreported toxin binding site (sections 4.3.2.1; 5.6.0) might be further investigated by doing some simple activity studies with intact nuclei (prepared by the method of Bloebel and Potter, 1966). One could examine whether intact cholera toxin or the B subunit of the toxin affects nuclear ADP ribosylation or another nuclear process. Again, the sensitive post-embedding immunogold technique could be used to localise internalised toxin molecules inside individual nuclei.

Precise biochemical analysis of nuclear fractions should be performed, perhaps by High Performance Liquid Chromatography; with ganglioside molecules as standards to examine the existence of such molecules in this cellular organelle. The major problem with extracting nuclei is that the procedure also allows contaminating cellular membranes to be extracted with the nuclei and this problem has to be overcome before a definitive answer can be sought.

6.2 GM_1 Ganglioside

Gangliosides are being increasingly studied, probably because of their possible involvement as cell surface markers for oncogenesis (section 1.2.3.2). They remain molecules whose structures have been elucidated but whose functions remain unknown.

6.2.1 GM_1 lateral mobility

The mobility of ganglioside GM_1 has been demonstrated on several cell types, (see table 3.1). In experiments described in this study GM_1 ganglioside mobility has also been demonstrated (see section 3.5.2). However, a new technique has emerged which allows individual cell components to be examined. This technique is 'nanovid tracking' (Geerts et al. 1987) (nanovid = nanopartide video microscopy). This technique enables the experimenter to follow the motion of individual colloidal gold markers on the cell.

If the colloidal gold were adsorbed onto a Fab fragment of a monoclonal antibody, specific for GM_1 ganglioside, then it follows that the motion of individual GM_1 ganglioside molecules on the cell could be observed. This procedure would also allow the fraction of mobile and immobile GM_1 ganglioside molecules to be examined.

6.2.2 Ganglioside Clusters

The existence of ganglioside clusters (section 3.6.2) needs to be further examined. Immunogold surface replica studies using a specific anti-ganglioside GM_1 antibody should be carried out. One might also be able to determine whether gangliosides exist as clusters by the use of photoactive cross-linking agents. Such agents were used to determine how far the subunits of cholera toxin penetrated the lipid bilayer of an artificial system (Wisnieski and Bramhall, 1981).

The work presented in this thesis shows how powerful a tool cholera toxin is for examining the location and possible function of GM_1 ganglioside in the cell and how much of its mechanism of action still remains to be elucidated.

REFERENCES

Abrahamsson, S., Dahlen, B., Lofgren, H., Pascher, I. & Sundell, S. (1977) in: "Structure of Biological Membranes", Abrahamsson, S. & Pascher, I., Eds., New York / London, Plenum, 1-23

Ackerman, G. A., Wolken, K. W. & Gelder, F. B. (1980) J. Histochem. Cytochem. 28, 1100-1112

Agutter, P. S. (1986) in: "The Nuclear Envelope and RNA Maturation", Smuckler, E. A. & Clawson, G. A., Eds., New York, Alan R. Liss, 561-578

Alessandri, G., Filippeschi, S., Sinibaldi, P., Mornet, F., Passera, P. & Sprecifico, F. (1987) Cancer Research 47, 4243-4247

Ando, S. & Yu, R. K. (1979) J. Biol. Chem. 254, 12224-12229

Aranda, A. & Samuels, H. H. (1984) J. Biol. Chem. 259, 6110-6116

Axelrod, D. (1977) Biophys. J. 18, 129-131

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. (1976) Biophys. J. 16, 1055-1069

Baeniziger, J. U. & Fiete, D. (1979) J. Biol. Chem. 254, 9795-9799

Basu, S., Basu, M., Chein, J. L. & Presper, K. A. (1979) Adv.Exp.Med.Biol. 125, 213-226

Basu, S., Moskal, J. R. & Gardner, D. A. (1976) in: "Ganglioside Function:Biochemical and Pharmocological Implications", Porcellati, G., Ceccarelli, B. & Tettamanti, G., Eds., New York, Plenum, 45

Bauer, H., Gerber, H. & Horisberger, M. (1975) Experientia. 31, 1149-1151

Beesley, J. E. (1985) Proc. R.M.S. 20, 187-196

Bennett, V. & Cuatrecasas, P. (1975) J. Membr. Biol. 22, 29-52

Bennett, V. & Cuatrecasas, P. (1976) J.Supermol.Struct. 4, 99-120

Bloebel, G. & Potter, V. R. (1966) Science 154, 1662-1665

Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L. & Gilman, A. G. (1983) J. Biol. Chem. 258, 2072-2075

Bourguignon, L. Y. W. & Singer, S. J. (1977) J.Cell Biol. 75, 216A

Bradford, M. M. (1976) Anal.Biochem. 72, 248-254

Brady, R. O. & Fishman, P. H. (1974) Biochim. Biophys. Acta 355, 121-148

Bretscher, M. S. (1976) Nature, London 260, 260-21

Burwen, S. J. & Jones, A. L. (1987) Trends Biochem. Sci. 12, 159-162

Cassel, D., Levkovitz, H. & Selinger, Z. (1977) J. Cyclic Nucleotide Res. 3, 393-406

Cassel, D. & Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2669-2673

Cherry, R.J. (1979) Biochim. Biophys. Acta 559, 289-327

Chou, P. Y. & Fasman, G. D. (1978) Ann. Rev. Biochem. 41, 251-276

Clements, J. D. & Finkelstein, R. A. (1978) Infect. Immun 21, 1036-1039

Clements, J. D. & Finkelstein, R. A. (1979) Infect. Immun. 24, 760-769

Clements, J. D., Yamay, R. J. & Finkelstein, R. A. (1980) Infect. Immun. 29, 91-97

Collier, R. J. (1975) Bacteriol.Rev. 39, 54-85

Coons, A. H. (1941) Proc. Soc. Exp. Biol. Med. 47, 200-202

Craig, S. & Cuatrecasas, P. (1976) Proc. Natl. Acad. Sci. U.S.A. 72, 2669-2673

Critchley, D. R., Mangani, J. L. & Fishman, P. H. (1981) J. Biol. Chem. 256, 8724-8731

Cuatrecasas, P. (1973a) Biochemistry 12, 3547-3558

Cuatrecasas, P. (1973b) Biochemistry 12, 3558-3566

Cuatrecasas, P. (1973c) Biochem. 12, 3567-3577

Dahlqvist, A. (1968) Anal. Biochem. 22, 99-107

Davison, M. T. & Garland, P. B. (1983) Eur. J. Biochem. 130, 589-597

De Roe, C., Coutoy, P. J. & Baudhuim, P. (1987) J. Histochem. Cytochem. 35, 1191-1198

De, S. N. (1961) "Cholera, its Pathology and Pathophysiology", Edinburgh/London, Oliver and Boyd, 380-387

De Wolf, M. J. S., Fridkin, M. & Kohn, L. D. (1981) J. Biol. Chem. 256, 5489-5496

Dixon, S.J., Stewart, D., Grinstein, S. & Speigel, S. (1987) J. Cell Biol. 105, 1153-1161

Dnistran, A. M., Skipski, V. P., Barclay, M. & Stock, C. C. (1979) J. Natl. Cancer Inst. 62, 367-370

Donta, S. T., Poindexter, N. J. & Ginsberg, B. H. (1982) Biochem. 21, 660-664

Douglas, M. & Butow, R. A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1083-1086

Dragsten, P., Kenkart, P., Blumenthal, R., Weinstein, J. & Schlessinger, J. (1979) Proc. Natl. Acad. Sci. USA 76, 5163-5167

Dreyfus, H., Urban, P. F., Edel-Harth, S. & Mandel, P. (1975) J. Neurochem. 25, 245-250

Duffy, L. K. & Lai, C. Y. (1979) Biochem. Biophys. Res. Commun. 91, 1005-1010

Duffy, L. K., Peterson, J. N. & Kurosky, A. (1981) FEBS Lett. 126, 182-190

Dulbecco, R. & Freeman, G. (1959) Virology 8, 396-397

Dykes, C. W., Halliday, I. J., Hobden, A. N., Read, M. J. & Harford, S. (1985) FEMS Microbiol. Lett. 26, 171-174

Edidin, M. (1981) in: "Membrane structure and function", New Comprehensive Biochemistry vol.1, Finean, J. B. & Michell, R. H., Eds., Amsterdam, Elsevier, 37-82

Edidin, M. (1987) in: "Current topics in Membranes and Transport", vol.29, Klausner, R. D., Kempf, C. & van Renswoude, J., Eds., New York, Academic Press, 91-127

Eidels, L., Proia, R. L. & Hart, D. A. (1983) Microbiol. Rev. 48, 199-221

Eldridge, C. A., Elson, E. L. & Webb, W. W. (1980) Biochem. 19, 2075-2079

Endo, Y., Mitsui, K., Motizuki, M. & Tsurugi, K. (1987) J. Biol. Chem. 262, 5908-5912

Evans, W. H. & Bergeron, J. J. M. (1988) Trends Biochem. Sci. 13, 7-8

Facci, L., Skaper, S. D., Favaron, M. & Leon, A. (1988) J. Cell. Biol. 106, 821-828

Faulk, W. P. & Taylor, G. M. (1971) Immunochem. 8, 1081-1083

Feizi, T. (1985) Nature London 314, 314-53

Finkelstein, R. A., LaRue, M. K. & LoSpalluto, J. J. (1972) Infect.Immun. 6, 934-935

Fishman, P. H. (1980) J. Membr. Biol. 54, 61-72

Fishman, P. H. (1982) J. Membr. Biol. 69, 85-97

Fishman, P. H. & Atikaan, E. E. (1979) J. Biol. Chem. 254, 4342-4344

Fishman, P. H. & Atikaan, E. E. (1980) J. Memb. Biol. 54, 51-60

Fishman, P. H., Bradley, R. M., Hom, B. E. & Moss, J. (1983) J.Lipid Res. 24, 1002-1011

Fishman, P. H. & Brady, R. O. (1976) Science 194, 906-915

Fishman, P. H., Moss, J. & Osbourne, J. C. (1978) Biochemistry 17, 711-716

Fishman, P. H., Moss, J. & Vaughan, M. (1976) J. Biol. Chem. 251, 4490-4494

Fitsgerald, D., Morris, R. E. & Saelinger, C. B. (1980) Cell 21, 867-873

Foley, M., Macgregor, A. N., Kusel, J. R., Garland, P. B., Downie, T. & Moore, I. (1986) J. Cell. Biol. 103, 807-818

Franke, W. W. (1974) Int. Rev. Cytology S4, 72-236

Freddo, L., Hays, A. P., Nickerson, K. G., Spatz, L., McGinnis, S., Lieberson, R., Vedeler, C. A., Shy, M. E., Autilio-Gambetti, L., Grauss, F. C., Petito, F., Chess, L. & Latov, N. (1986) J. Immunol. 137, 3821-3825

Frens, G. (1973) Nature Phys. Sci. 241, 20-22

Frye, L. D. & Edidin, M. (1970) J.Cell Science 7, 319-335

Garfield, E. (1986) Current Contents 14, 3-118

Garland, P. B. (1981) Biophys.J. 33, 481-482

Geerts, H., De Brabander, M., Nuydens, R., Gueens, S., Moremans, M., De Mey, J. & Hollenbeck, P. (1987) Biophys. J. 52, 775-782

Geoghegan, W. D. & Ackerman, G. A. (1977) J. Histochem. Cytochem. 25, 1187-1200

Gerace, L., Ottaviano, Y. & Kondor-Koch, C. (1982) J. Cell. Biol. 95, 826-837

Gill, D. M. (1975) Proc. Natl. Acad. Sci. U.S.A. 78, 152-156

Gill, D. M. (1976) J. Infect. Dis. 133, s55-s63

Gill, D. M. (1977) Adv.Cyc.Nuc.Res. 8, 85-118

Gill, D. M. (1979) J. Supermol. Struct. 10, 151-163

Gill, D. M., Clements, J. D., Robertson, D. C. & Finkelstein, R. A. (1981) Infect. Immun. 33, 677-682

Gill, D. M. & King, C. A. (1975) J. Biol. Chem. 250, 6424-6432

Gill, D. M. & Meren, R. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3050-3054

Gill, D. M. & Rappaport, R. (1979) J. Infect. Dis. 139, 674-680

Gill, D. M. & Richardson, S. H. (1980) J.Infect.Dis. 141, 64-70

Goding, J. W. (1983) "Monoclonal Antibodies, Principles and Practice", Academic Press, 208-230

Goins, B., Maserini, M., Barisas, B. G. & Friere, E. (1986) Biophys.J. 49, 849-856

Goodman, S. L., Hodges, G. M., Trejdosiewicz, L. K. & Livingston, D. C. (1981) J. Microscopy 123, 201-213

Greenhough, W. B. III, Pierce, N. F. & Vaughan, M. (1970) J. Infect. Dis. 121, S111-S113

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) Biochem. J. 89, 114-123

Griffiths, S. L., Fikelstein, R. A. & Critchley, D. R. (1986) Biochem. J. 238, 313-322

Hakomori, S. I. (1966) J.Lipid Res. 7, 789-792

Hakomori, S. I. (1975) Biochim. Biophys. Acta 417, 55-89

Hakomori, S. I. (1981) Ann.Rev.Biochem. 50, 733-764

Hakkinen, H. M. & Kulonen, (1963) Nature London 198, 995

Hanai, N., Nores, G., Mendez, C. R. T. & Hakomori, S. I. (1987) Biochem. Biophys. Res. Commun. 1, 127-134

Hansson, H. A., Holmgren, J. & Svennerholm, (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3782-3786

Harris, J. R. & Milne, J. F. (1974) Biochem. Soc. Transactions 2, 1251-1253

Hart, G. W., Holt, G. D. & Haltiwanger, R. S. (1988) Trends Biochem. Sci. 13, 380-384

Haugland, R.P. (1989) "Handbook of Fluorescent Probes and Research Chemicals", Eugene, Molecular Probes, 32

Holmes, R.K. & Twiddy E.M. (1983) Infect. Immun. 42, 914-923

Holmgren, J., Elwing, H., Fredman, P. & Svennerholm, L. (1980) Eur. J. Biochem. 106, 371-379

Holmgren, J., Lindolm, L. & Lonroth, I. (1974) J. Exp. Med. 139, 801-819

Holmgren, J., Lonroth, I., Mansson, J. E. & Svennerholm, L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2520-2524

Holmgren, J., Lonroth, I. & Svennerholm, L. (1973) Infect. Immun. 8, 208-214

Horisberger, M. (1981) in: "Scanning Electron Microscopy", Jahari, O., Ed., Chicago, O'Hare, 9-31

Horisberger, M. & Rosset, J. (1977) J. Histochem. Cytochem. 25, 295-305

Houslay, M. D. & Elliot, K. R. F. (1981) FEBS Lett. 128, 289-292

Hyun, C. S. & Kimmich, G. A. (1984) Amer. J. Physiol. 247, G623-G631

Imboden, J. B., Shoback, D. M., Pattison, G. & Stobo, J. D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5673-5677

Irons, L. I. & Maclennan, A. P. (1979) Biochim. Biophys. Acta 580, 175-185

Jacobs, J. W., Niall, H. D. & Sharp, G. W. G. (1974) Biochem. Biophys. Res. Commun. 61, 391-395

Jacobson, K., Hou, Y. & Wojcieszyn, J. (1978) Exp. Cell Res. 116, 179-189

Jacobs, S. & Cuatrecasas, P. (1977) Trends Biochem. Sci. 2, 280-282

Janicot, M. & Desbuquois, B. (1987) Eur. J. Biochem. 163, 433-442

Johnson, V. G., Wilson, D., Greenfield, L. & Youle, R. J. (1988) J. Biol. Chem. 263, 1295-1300

Johnstone, A. (1986) in: "Immunocytochemistry in Practice", Johnstone, A. & Thorpe, R., Eds., Oxford, Blackwell Scientific, 44-45

Joseph, K. C., Steiber, A. & Gonatas, N. K. (1979) J. Cell. Biol. 81, 543-554

Kauffmann, S. H., Gibson, W. & Shaper, J. H. (1983) J. Biol. Chem. 258, 2710-2719

Keenan, T. W., Huang, C. M. & Morre, D. J. (1972a) Biochem. Biophys. Res. Commun. 47, 1277-1283

Keenan, T. W., Morre, D. J. & Basu, S. (74c) J. Biol. Chem. 249, 310-346

Keenan, T. W., Morre, D. J. & Huang, C. M. (1972b) FEBS Lett. 24, 204-208

Kellie, S., Patel, B., Pierce, E. J. & Critchley, D. R. (1983) J.Cell Biol. 97, 447-454

King, C. A. & van Heyningen, W. E. (1973) J. Infect. Dis. 127, 639-647

King, C. A., van Heyningen, W. E. & Gascoyne, N. (1976) J. Infect. Dis. 133, 575-581

Kitamura, M., Iwamori, M. & Nagai, Y. (1980) Biochim. Biophys. Acta 628, 328-335

Klenk, E. (1935) Hoppe-Seylers Z. Physiol. Chem. 235, 24-36

Klenk, E. (1942) Hoppe-Seylers Z. Physiol. Chem. 273, 76-86

Klotz, I. M. (1986) "Introduction to Biomolecular Energetics", London, Academic Press, 103-133

Kuhn, R. & Wiegandt, H. (1963) Chem. Ber. 86, 866-880

Kurosky, A., Markel, D. E., Peterson, J. W. & Fitch, W. M. (1977) Science 195, 299-301

Laemmli, U. K. (1970) Nature London, 227, 680

Lai, C. Y. (1980) C.R.C. Crit. Rev.Biochem 9, 171-205

Lai, C. Y., Cancedda, F. & Chang, D. (1979) FEBS Lett. 100, 85-89

Lai, C. Y., Mendez, E., Chang, D. & Wang, M. (1977) Biochem. Biophys. Res. Commun. 74, 215-222

Landsteiner, K. & Levene, P. A. (1925) J. Immunol. 10, 731-733

Ledeen, R. W. (1983) in: "Handbook of Neurochemistry", Lajthia, A., Ed., 15-90

Ledeen, R. W. & Yu, R. K. (1982) in: "Methods in Enzymology", Ginsburg, V., Ed., New York, Academic Press, 83, 139-191

Ledley, F. D., Mullin, B. R., Lee, G., Aloj, S. M., Fishman, P. H., Hunt, L. T., Dayhoff, M. O. & Kohn, L. D. (1976) Biochem. Biophys. Res. Commun. 69, 852-859

Lindolm, L., Holmgren, J., Wikstrom M, Karlsson, U., Anderson, K. & Lycke, N. (1980) Infect.Immun. 40, 570-576

Lin, M. C. & Taniuchi, M. (1980) J. Cyclic. Nucleotide. Res. 6, 359-367

Lipsky, N. G. & Pagano, R. E. (1985) J. Cell. Biol. 100, 27-34

Longbottom, D. & van Heyningen, S. (1989) Biochim. Biophys. Acta 1014, 289-297

LoSpalluto, J. J. & Finkelstein, R. A. (1972) Biochim. Biophys. Acta 257, 158-166

Ludwig, D. S., Ribi, H. O., Schoolnik, G. K. & Kornberg, R. D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 8585-8588

Marquardt, D. W. (1963) J. Soc. Ind. Applied Math. 11, 431-441

Matuo, Y., Wheeler, M. A. & Bitensky, M. W. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2654-2658

Matyas, G. R. & Morre, D. J. (1987) Biochim. Biophys. Acta 921, 599-619

Mekalanos, J. J., Collier, J. J. & Romig, W. R. (1979) J. Biol. Chem. 254, 5855-5861

Mekalanos, J. J., Swartz, D. J., Pearson, G. D., Harford, N., Groyne, F. & De Wilde, M. (1983) Nature London 306, 306-551

Mellanby, J. & Green, J. (1981) Neuroscience 6, 281-300

Miller-Prodrasa, H., Bradley, R. M. & Fishman, P. H. (1982) Biochemistry 21, 3260-3265

Moller, G. (1961) J.Exp.Med. 114, 417-419

Montesano, R., Roth, J., Robert, A. & Orci, L. (1982) Nature London 296, 296-651

Morita, A., Tsao, D. & Kim, Y. S. (1980) J. Biol. Chem. 255, 2549-2553

Mosley, S. L. & Falkow, S. (1980) J. Bact. 144, 444-446

Moss, J., Fishman, P. H., Manganiello, V. C., Vaughan, M. & Brady, R. O. (1976a) Proc. Natl. Acad. Sci. U.S.A. 73, 3480-3483

Moss, J., Fishman, P. H., Manganiello, V. C., Vaughan, M. & Brady, R. O. (1976b) Proc. Natl. Acad. Sci. U.S.A. 73, 1034-1037

Moss, J., Gamson, S., Fishman, P. H. & Richardson, S. H. (1979) J. Clin. Invest. 64, 381-384

Moss, J., Osbourne, J. C., Fishman, P. H., Brewer, H. B., Vaughn, M. & Brady, R. O. (1977a) Proc. Natl. Acad. Sci. (USA) 74, 74-78

Moss, J., Osbourne, J. C., Fishman, P. H., Nagaya, S. & Robertson, D. C. (1981) J. Biol. Chem. 256, 12861-12865

Moss, J., Richards, R. L., Alving, C. R. & Fishman, P. H. (1977b) J. Biol. Chem. 52, 79-83

Moss, J. & Vaughan, M. (1977) J. Biol. Chem. 252, 2455-2457

Mullin, B. B., Aloj, S. M., Fishman, P. H., Lee, G., Kohn, L. D. & Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1679-1683

Nagai, Y. & Iwamori, M. (1980) Mol. Cell. Biochem. 96, 866-880

Nakane, P. K. & Pierce, G. B. (1966) J. Histochem. Cytochem. 14, 929-931

Nakashima, Y., Napiorkowski, P., Shafer, D. E. & Konigsberg, W. H. (1976) FEBS Lett. 68, 275-278

Ohsawa, T., Ikeda, H. & Senshu, T. (1988) Biochim. Biophys. Acta 949, 305-310

Olsnes, S. & Pihl, A. (1976) in: "The specificity and action of animal, bacteria and plant toxins", Cuatrecasas, P., Ed., London, Chapman and Hall, 131

Olsnes, S. & Pihl, A. (1982) in: "The molecular action of toxins and viruses", Cohen, P. & van Heyningen, S., Eds., Amsterdam, Elsevier, 51-105

Olsnes, S. & Sandvig, K. (1986) J. Biol. Chem. 261, 1542-1552

Ouchterlony, O. (1958) Prog. Allergy 5, 1-78

Pappenheimer, A. M. (1977) Ann. Rev. Biochem. 46, 69-94

Pardoe, G. I., Bird, G. W. G. & Uhlenbrook, G. (1969) Z. Immunitals. Forsch. 137, 442-457

Pastan, I. H. & Willingham, M. C. (1981) Science 214, 504-509

Peters, R. (1981) Cell Biol.Int. Reports 5, 733-759

Peters, R. (1988) FEBS Lett. 234, 1-7

Peters, R., Peters, J., Tews, K. H. & Bahr, W. (1974) Biochim.Biophys.Acta 367, 282-294

Peters, R. & Richter, H. P. (1981) Develop. Biol. 86, 285-293

Pfeuffer, T. (1977) J. Biol. Chem. 252, 7224-7234

Pierce, N. F. (1973) J. Exp. Med. 137, 1009-1023

Ploem, J. S. (1967) Z.Wiss.Mikrosk. 68, 129-142

Pollitzer, R. (1965) Proceedings of the Cholera Research Symposium, Honolulu Hawaii, 380-387

Poo, M. & Cone, R. (1974) Nature London 247, 438-441

Reed, B. C., Moss, J., Fishman, P. H. & Lane, M. D. (1980) J. Biol. Chem. 255, 1711-1715

Revesz, T. & Greaves, M. (1975) Nature London 257, 257-103

Ribi, H. O., Ludwig, D. S., Mercer, L., Schoolnik, G. K. & Kornberg, R. D. (1988) Science 239, 1272-1276

Rodbell, M. (1980) Nature London, 284-17

Rohlich, P. & Allison, A. C. (1976) J. Ultrastructure Res. 57, 94-103

Roseman, S. (1970) Chem.Phys.Lipids 5, 270-297

Ross, E. M. & Gilman, A. G. (1980) Ann. Rev. Biochem. 49, 533-564

Roth, J. (1982) in: "Techniques in Immunocytochemistry", Bullock, G. R. & Petrusz, P., Eds., London, Academic Press, 107-134

Roth, J. (1983) in: "Techniques in Immunocytochemistry", vol 2, Bullock, G. R. & Petrusz, P., Eds., London, Academic Press, 315-321

Roth, J. (1985) Glycoconjugate J. 2, 315-321

Roth, J. & Binder, M. (1978) J.Histochem. Cytochem. 26, 163-169

Saffman, P. G. & Delbruck, (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3111-3113

Sahyhoun, N. & Cuatrecasas, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3438-3442

Sahyhoun, N., Shatila, T., Levine, H. III & Cuatrecasas, P. (1981) Biochim. Biophys. Res. 102, 1216-1222

Salamon, Y. (1979) Adv. Cyc. Nucl. Res. 10, 35-55

Sandvig, K., Olsnes, S. & Pihl, A. (1976) J. Biol. Chem. 251, 3977-3984

Sattler, J., Wiegandt, H., Sterk, J., Kranz, T., Ronneberger, H. J., Schmidtberger, R. & Zilge, H. (1975) Eur.J.Biochem. 57, 309-316

Scatchard, G. (1949) Ann. NY. Acad. Sci. 51, 660-672

Schlessinger, J., Axelrod, D., Koppel, D. E., Webb, W. W. & Elson, E. L. (1977a) Science 195, 307-309

Schlessinger, J., Barak, L. S., Hammes, G. G., Yamodo, K. M., Pastan, I., Webb, W. W. & Elson, E. L. (1977b) Proc. Natl. Acad. Sci. U.S.A. 74, 2909-2913

Schlessinger, J., Webb, W. W., Elson, L. E. & Metzger, H. (1976) Nature London 264, 550-552

Schreiber, A. B. & Haimovich, J. (1983) Meths. in Enzymol. 93, 147-155

Sedlacek, H. H., Staerk, J., Seiler, F. R., Ziegler, W. & Wiegandt, H. (1976) FEBS Lett. 61, 272-276

Sharom, F. J. & Grant, C. W. M. (1977) J.Supramol.Struct. 6, 249-258

Sharom, F. J. & Grant, C. W. M. (1978) Biochim. Biophys. Acta 507, 280-293

Shur, B. D. & Roth, S. (1975) Biochim. Biophys. Acta 415, 473-512

Sigler, P. B., Druyan, M. E., Kiefer, H. C. & Finkelstein, R. A. (1977) Science 197, 1277-1279

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Konigsberg, W. H. & Schafer, D. (1981) J. Biol. Chem. 256, 1094-1097

Sinai, A. B., Aldouby, Y., Chorev, M. & Levitski, A. (1986) EMBO Journal 5, 1175-1180

Singer, S. J. & Nicolson, G. L. (1972) Science 175, 720-731

Skiar, L. A., Doody, M. C., Gotto, A. M. & Pownall, H. H. (1980) Biochemistry 19, 1294-1301

Snow, C. M., Senior, A. & Gerace, L. (1987) J. Cell. Biol. 104, 1143-1156

Spicer, E. K., Kavanaugh, W. M., Dallas, W. S., Falkow, S., Konigsberg, W. H. & Schafer, D. E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 50-54

Spicer, E. K. & Noble, J. A. (1982) J. Biol. Chem. 257, 5716-5721

Spiegel, S. (1985) Biochem. 24, 5947-5952

Spiegel, S. (1987) Methods in Enzymology 138, 313-318

Spiegel, S. (1988) Biochimica et Biophysica Acta 969, 249-256

Spiegel, S. & Fishman, P. H. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 141-145

Spiegel, S., Kassis, S., Wilchek, M. & Fishman, P. H. (1984) J.Cell Biol. 99, 1575-1581

Spiegel, S., Mayyas, G. R., Cheng, L. & Sacktor, B. (1988) Biochim. Biophys. Acta 938, 270-278

Spiegel, S., Wilchek, M. & Fishman, P. H. (1983) Biochem. Biophys. Res. Commun. 112, 872-877

Staerk, J., Ronneberger, H. J., Wiegandt, H. & Ziegler, W. (1974) Eur. J. Biochem. 48, 103-110

Stoffel, W. (1971) Ann. Rev. Biochem. 40, 57-82

Streuli, C. H., Patel, B. & Critchley, D. R. (1981) Exp.Cell. Res. 136, 247-254

Sugiyama, H. (1980) Mirobiol.Rev. 44, 419-448

Suzuki, K. (1965) J. Neurochem 12, 969-979

Svennerholm, A. M. & Holmgren, J. (1978) Curr. Microbiol. 1, 19-23

Svennerholm, L. (1963) J.Neurochem. 10, 613-623

Svennerholm, L. (1970) in: "Comprehensive Biochemistry" vol. 18, Florkin, M. & Stotz, E. H., Eds., Amsterdam, Elsevier, 201-227

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. & Ishii, S. (1982) Biochem. 21, 5516-5522

Taylor, R. B., Duffus, W. P. H., Raff, M. C. & de Petris, S. (1971) Nature New Biol. 233, 225-229

Tettamenti, G., Bertona, L. & Zambetti, V. (1964) Biochim. Biophys. Acta 84, 756-758

The, T. H. & Feltkamp, T. E. W. (1970a) Immunology 18, 865-873

The, T. H. & Feltkamp, T. E. W. (1970b) Immunology 18, 875-881

Thiessen, P. A. (1942) Kolloid Z. 101, 242-248

Thompson, L. K., Horowitz, P. M., Bentley, K. L., Thomas, D. D., Aldrete, J. F. & Klebe, R. J. (1986) J. Biol. Chem. 261, 5209-5214

Thompson, N. L. & Axelrod, D. (1981) Biophys. J. 33, 435-454

Tomasi, M. & Montecucco, C. (1981) J. Biol. Chem. 256, 11177-11181

Tonegawa, Y. & Hakomori, S. (1977) Biochem. Biophys. Res. Commun. 76, 9-17

Tosteson, M. T. & Tosteson, D. C. (1978) Nature London 275, 142-144

Towbin, H., Staehlin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354

Uchida, T. (1982) in: "The molecular action of toxins and viruses", Cohen, P. & van Heyningen, S., Eds., Amsterdam, Elsevier, 1-31

van Heyningen, S. (1976) J.Infect.Dis 133, s5-s13

van Heyningen, S. (1977) Biochem. J. 168, 457-463

van Heyningen, S. (1980) Pharmacol.Ther. 11, 141-157

van Heyningen, S. (1982) Eur. J. Biochem. 122, 333-337

van Heyningen, S. (1983) Current Topics in Membranes and Transport 18, 445-471

van Heyningen, S. & King, C. A. (1975) Biochem. J. 146, 269-271

van Heyningen, S. & Tait, R. M. (1980) in: "Hormones and cell regulation" vol. 4, Dumont,J., & Nunez, I., Eds, Amsterdam, Elsevier, 293-309

van Heyningen, W. E., Carpenter, C. C., Pierce, N. F. & Greenhough, W. B. (1971) J. Infect. Dis. 124, 415-418

Varndell, I. M. & Polak, J. M. (1986) in: "Immunocytochemistry: Modern Eethods and Applications", 2nd ed., Polak, J. M. & Van Noorden, S., Eds., Bristol, Wright, 146-166

Walz, E. (1927a) Hoppe-Seylers Z. Physiol. Chem. 166, 210-222

Walz, E. (1927b) Hoppe-Seylers Z. Physiol. Chem. 166, 223-226

Ward, W. H. J., Britton, P. & van Heyningen, S. (1981) Biochem. J. 199, 457-460

Weir, M. L. & Edidin, M. (1986) Journal of Cell Biology 103, 215-222

White, A. A. (1974) Methods in Enzymology 38, 41-46

Wisnieski, B. J. & Bramhall, J. S. (1981) Nature London 289, 319-321

Wolf, D.E., Hankart, P. & Webb, W.W. (1980) Biochem. 19, 3893-3895

Wood, B. T., Thompson, S. H. & Goldstein, G. (1965) J. Immunol. 95, 225-228

Yamakawa, T. & Nagai, Y. (1978) Trends Biochem. Sci. 3, 128-131

Yamakawa, T. & Susuki, S. (1951) J. Biochem. (Tokyo) 38, 199-212

Yguerabide, J., Schmidt, A. & Yguerabide, E. (1982) Biophys. J. 39, 69-75

Zierler, K. (1989) Trends. Biochem. Sci. 214, 314-317

Zieve, P. D., Pierce, N. F. & Greenhough, W. B. III (1971) Johns Hopkins Med. J. 129, 299

Zsigmondy, R. (1901) Z. Analyt. Chem. 40, 697-719

Fehrenbach et al. (Eds.), Bacterial Protein Toxins, Zbl. Bakt. Suppl. 17

Localisation of a Cholera Toxin Binding Component in the Nuclei of Mouse Intestinal Cells by the Post Embedding Immunogold Technique.

M. E. Parkinson[1], C. G. Smith[2], S. van Heyningen[1] and P. B. Garland[2]

[1]Department of Biochemistry, University of Edinburgh, Hugh Robson Building, George Square, EH8 9XD. Scotland
[2]Unilever Research, Colworth House, Sharnbrook, Bedford. MK44 1LQ, U.K.

ABSTRACT

A cholera toxin binding component (probably GM1 Ganglioside) has been located in the chromatin of intestinal cell nuclei. Cholera toxin binds with high affinity ($Kd=10^{-9}M$) and specificity to ganglioside GM1. Localisation involved a sandwich of cholera toxin, antitoxin antibody and gold labelled second antibody on mouse intestine, previously embedded in hydrophilic resin. There was a sparse distribution of gold amongst the microvilli, clusters in the cytoplasm, in vesicle-like structures and most surprisingly, a distribution of gold accross the chromatin of the nuclei. All binding could be abolished by preincubating the toxin with ganglioside GM1. Anti-GM1 antibody also bound to the nucleus, indicating the presence of the ganglioside or a similar molecule in the nucleus.

KEYWORDS

cholera toxin, GM1 ganglioside, mouse intestine, post embedding immunogold

INTRODUCTION

Previous studies on the immunocytochemical localisation of GM1 ganglioside used peroxidase conjugated anti-cholera toxin antibody (Hansson, 1977), or the preembedding immunogold technique (Ackerman, 1980). However, both these studies have concentrated on the external GM1 molecules located at the outer surface of the plasma membrane. The only evidence for the intracellular location of GM1 has been demonstrated by biochemical fractionation techniques (Keenan, 1972). Cholera toxin has been shown to bind to GM1 almost exclusively, on the cell surface (Hollenberg,1974). Therefore cholera toxin is a natural probe for GM1 ganglioside and using it to locate GM1 intracellularly may give some indication of the physiological role of the ganglioside. Some gangliosides have altered expression in cancer cells (Feizi, 1985) implicating them in growth regulation. Recently, it has been shown, using cholera toxin B subunit (the ganglioside binding site), that the ganglioside may be a bimodal growth regulator of cells, acting synergistically with EGF (Speigel , 1987). Therefore, the possible location of GM1 ganglioside in the nuclei of mouse intestinal cells may reflect some aspect of this physiologically observed action.

RESULTS

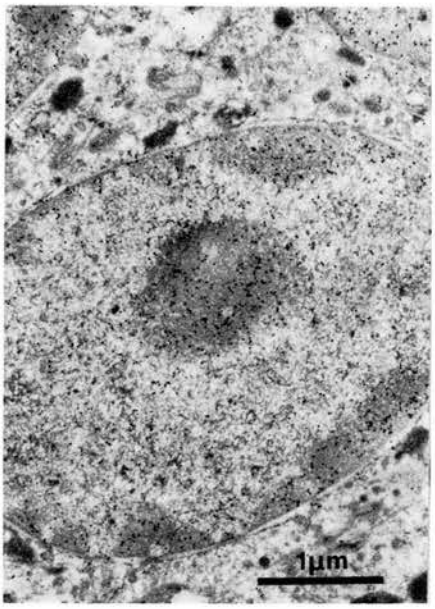


Fig.1. The binding of the B subunit of cholera toxin (1µg/ml in Dulbeccos PBS pH 7.4)

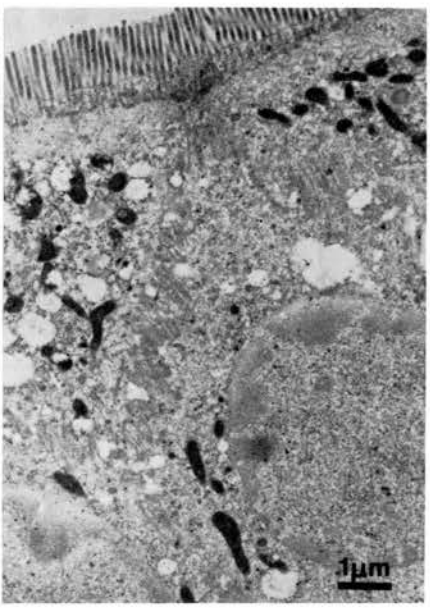


Fig.2. The binding of anti -GM1 antibody (1/100 in PBS buffer)

The results indicate that the predominant binding site for the cholera toxin is the heterochromatin near the nuclear envelope. Prior treatment with pronase abolishes this chromatin binding site. Preabsorption of the toxin with GM1 ganglioside abolishes all binding on both the nucleus and microvilli. Further work is underway to identify this cholera toxin binding component within the nucleus.

REFERENCES

Ackerman, G. A., K. W. Wolken, and F. B. Gelder. (1980). Surface Distribution of Monosialoganglioside GM1 on Human Blood Cells and the Effect of Exogenous GM1 and Neuraminidase on Cholera Toxin Surface labelling. J. Histochem. Cytochem., 28, 1100-1112.

Feizi, T. (1985). Demonstration by Monoclonal antibodies that Carbohydrate Structures of Glycoproteinsand Glycolipids are Onco-Developmental antigens. Nature, 314, 53-57.

Hansson, H. A., J. Holmgren, and L. Svennerholm. (1977). Ultrastructural Localisation of Cell Membrane GM1 Ganglioside by Cholera Toxin. Proc. Natl. Acad. Sci. U. S. A., 74, 3782-3786.

Hollenberg, M. D., P. H. Fishman, V. Bennet, and P. Cuatrecasas. (1974). Cholera Toxin and Cell Growth; Role of Membrane Gangliosides. Proc. Natl. Acad. Sci. U. S. A., 71, 4224-4228.

Keenan, T. W., D. J. Morre and C. M. Huang. (1972). Distribution of Gangliosides among subcellular Fractions from Rat Liver and Bovine mammary Gland. Febs. Letts., 24,204-208.

Speigel, S and P. H. Fishman. (1987). Gangliosides as Bimodal Regulators of Cell Growth. Proc. Natl. Acad. Sci. 84, 141-145.

Volume 242, number 2, 309–313 FEB 06653 January 1989

Identification of cholera toxin-binding sites in the nucleus of intestinal epithelial cells

Marianne E. Parkinson, Colin G. Smith[+], Peter B. Garland[°] and Simon van Heyningen

Department of Biochemistry, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland, [+]Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ and *[°]Amersham International plc, Pollards Wood, Little Chalfont, England*

Received 28 October 1988

Post-embedding immunogold electron microscopy shows several binding sites for cholera toxin in mouse intestinal epithelial cells, particularly in the heterochromatin of the nucleus as well as in the plasma membrane. Anti-ganglioside GM1 antibodies also bound to the nucleus, but did not interfere with the binding of toxin. ^{125}I-labelled toxin bound specifically to a nuclear preparation from rabbit intestinal cells.

Cholera toxin; Immunogold; Heterochromatin; Ganglioside GM1; (Nucleus)

1. INTRODUCTION

Cholera toxin (M_r 84000, for general review see [1]) is composed of one A subunit and five B subunits. The B subunits bind to the outer membrane of cells and, by a mechanism not yet understood, this leads to the entry into the cell of the A1 polypeptide of the A subunit which activates adenylate cyclase by catalysing the ADP-ribosylation of the regulatory G_s protein. The binding of the B subunits is almost exclusively to ganglioside GM1 in the outer membrane. Binding is tight ($K_d = 10^{-9}$ M) and specific: cholera toxin has often been used as a marker for ganglioside GM1.

Previous studies in which the ganglioside GM1 was localized immunocytochemically have used toxin incubated with cells, followed by labelling with peroxidase-conjugated antitoxin [2] or with immunogold [3,4] before embedding. They have shown ganglioside GM1 only on the external membrane.

Correspondence address: S. van Heyningen, Department of Biochemistry, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland

In the experiments described in this paper we used the post-embedding immunogold method [5] to investigate binding sites for cholera toxin in epithelial cells of mouse small intestine. Labelling under these conditions shows binding sites (both intra- and extracellular) that are available to the toxin after the tissue had been fixed and sectioned: this does not imply that such sites would be accessible to the toxin in intact cells or in vivo. This technique has been used previously, for example, in investigating the intracellular localization of regulatory polypeptides [6]. We found binding sites for the toxin in the microvilli, in the plasma membrane, and in the heterochromatin of the nucleus.

2. MATERIALS AND METHODS

2.1. *Preparation of tissue for electron microscopy*

Small pieces of freshly excised mouse small intestine were fixed in 1% paraformaldehyde, 0.05% glutaraldehyde, 0.15 M NaCl, 50 mM phosphate buffer, pH 7.4, for 2 h at 4°C, washed in this buffer overnight at 4°C, dehydrated through ethanol, and embedded in hydrophilic resin (3 parts LR gold resin, London Resin Co.; 2 parts glycol methacrylate low acid, and 0.01% benzoin ethyl ester, Polysciences). The resin was polymerized

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

by ultra-violet radiation (360 nm) for 24 h at room temperature.

2.2. *Immunolabelling*

Ultrathin sections mounted on collodion-coated nickel grids were incubated with 0.5 M NH_4Cl, in a buffer containing 0.15 M NaCl, 50 mM phosphate, pH 7.0, for 1 h at room temperature to block free aldehyde groups; washed with several changes of buffer, and incubated first for 30 min in 1 mg/ml ovalbumin, and then for 30 min in 850 ng/ml cholera toxin (supplied by Sigma, and used at a concentration chosen experimentally to give specific labelling and a very low background). After several washes in buffer, they were incubated for 1 h in rabbit anti-(cholera toxin) serum [6] (diluted 250 times in the buffer), washed again and finally incubated for 30 min with goat anti-(rabbit IgG) labelled with 15 nm gold (Janssen Pharmaceuticals) diluted fifty times. All incubations were at 37°C. After further washings in buffer and then in water, the grids were counterstained in 2% uranyl acetate and lead citrate, and examined with a Jeol 100CX2 electron microscope.

2.3. *Preparation of a nuclear fraction*

Nuclei were prepared from rabbit intestinal epithelial cells (prepared from gut scrapings) by the method of Bloebel and Potter [8]. A preparation of nuclear envelope with associated heterochromatin was prepared from this by the method of Harris and Milne [9]. It was analysed by polyacrylamide gel electrophoresis in the presence of SDS, and shown to have the expected protein profile [10]. The plasma membrane-marker enzyme sucrase was assayed by the method of Dahlqvist [11].

2.4. *Measurement of toxin binding*

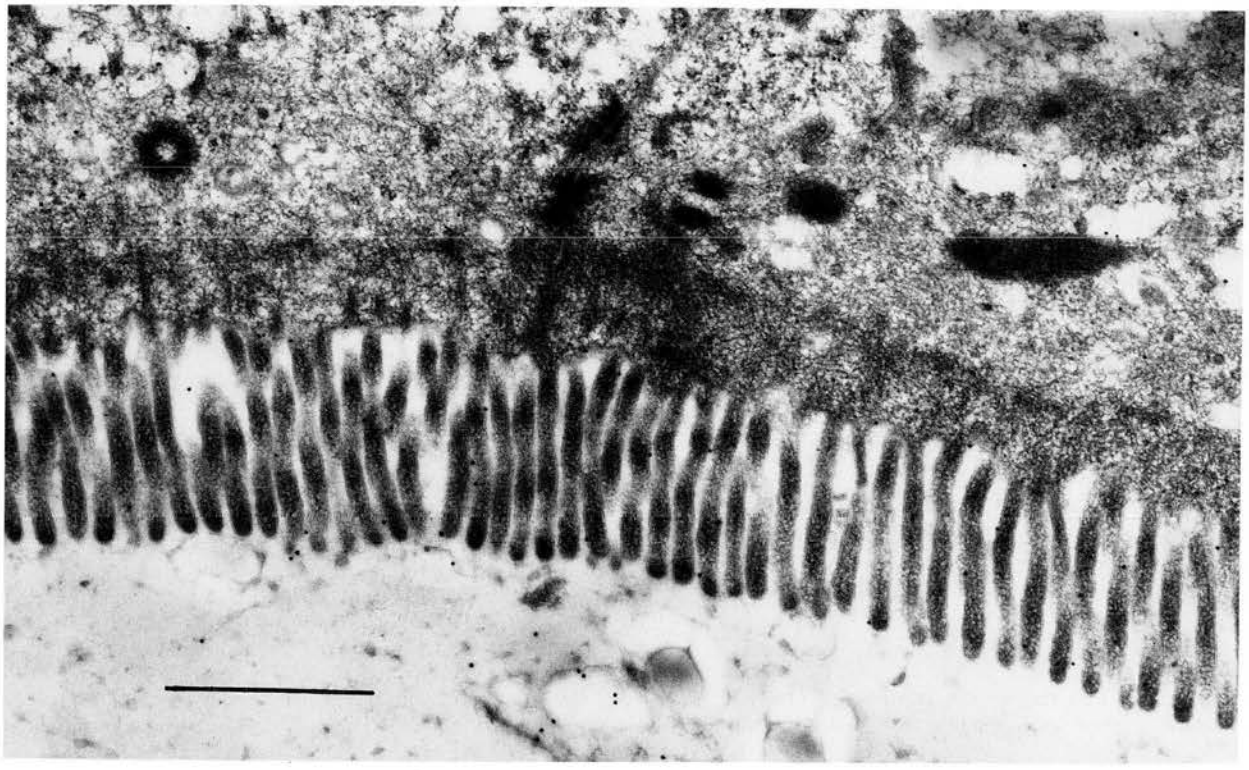
Cholera toxin was iodinated using chloramine-T [12], and used at about 70 GBq/μmol. Its binding to the nuclear preparation was measured essentially as described by Griffiths et al. [13] except that the buffer was 55 mM Tris-HCl, 80 mM NaCl, 10 mM mannose, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4. 250 μl of the nuclear preparation (400 μg/ml) were used for each triplicate determination. Controls using a forty-fold excess of unlabelled toxin were subtracted from experimental data. All duplicates showed less than 10% variation.

3. RESULTS

3.1. *Post-embedding immunogold electron microscopy*

Some results from the experiments designed to show binding sites for cholera toxin are shown in fig.1. There are two major sites, together with some low levels of binding elsewhere in the cell, e.g. to vesicular structures (perhaps lysosomes and coated vesicles) in the cytoplasm. As would have been predicted from earlier work, there was extensive binding of whole toxin or of isolated subunit

a



B to the plasma membrane and microvilli (as shown in fig.1a). More surprisingly, under the same experimental conditions, there was a heavy distribution of colloidal gold on the heterochromatin of the nuclei (fig.1b). These experiments were done with many different samples, and in all cases the major binding was to the plasma membrane and to the nucleus.

Controls were carried out to show that the observed nuclear binding was specific for toxin, and not, for example, to non-specific binding of the antibodies used in the immunogold labelling.

b

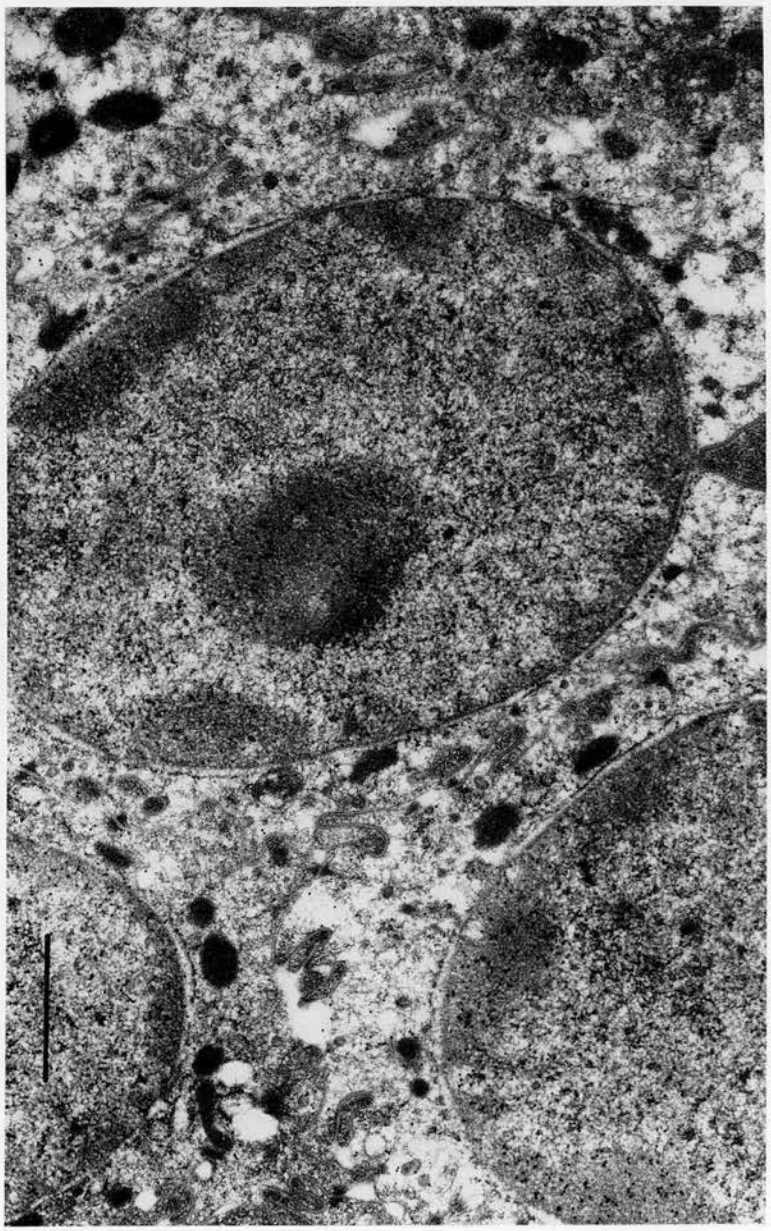


Fig.1. Electron micrographs of sectioned mouse intestinal tissue treated post-embedding with cholera toxin and immunogold. (a) Microvilli treated with 850 ng/ml whole toxin; (b) nuclei from the basal end of adjacent epithelial cells treated with 1 μg/ml subunit B. Bars are 1 μm long.

No gold was observed to bind to the nucleus or to the microvilli when (i) the rabbit antitoxin had been preadsorbed with toxin; (ii) cholera toxin was omitted; (iii) rabbit antitoxin was omitted; and (iv) the incubation was done with gold-labelled second antibody alone without toxin or second antibody.

Essentially identical binding was observed following similar experiments with rat intestinal cells.

3.2. *Are the binding sites ganglioside GM1?*

Several experiments were performed in order to find out whether the binding sites in the nuclei were ganglioside GM1. For example, toxin that had been preincubated with a 2×10^5-fold molar excess of ganglioside failed to bind, presumably because the binding site on the toxin was no longer available (although conceivably because of transmitted conformational change to a different binding site). Binding experiments done not with toxin but with a polyclonal anti-GM1 antibody preparation (kindly given to us by Dr N. Gregson of Guy's Hospital, London) gave very similar results to those found with toxin, suggesting a similar distribution of ganglioside and toxin-binding sites. However, preincubation of the post-embedded tissue with this antibody did not inhibit subsequent binding of toxin, nor did preincubation with toxin inhibit binding of antibody.

3.3. *Biochemical measurement of binding*

In order to verify that there are toxin-binding sites present in the nucleus, direct binding experiments were performed using ^{125}I-labelled toxin and a preparation of nuclear envelope from rabbit intestine.

Fig.2 shows a measurement of the binding of ^{125}I-labelled toxin to this preparation: the results are corrected for non-specific binding. Analysis of these data shows that they are compatible with a model in which there are about 4×10^{12} high-affinity binding sites (K_d about 2 nM) per mg protein, and about 6×10^{13} per mg of lower affinity (K_d about 70 nM).

Preliminary binding experiments using nuclear proteins adsorbed to microtitre plates gave similar results, and binding was abolished when the toxin was preincubated with ganglioside GM1. In order to establish the degree of contamination of the nuclear preparation with plasma membrane, it was

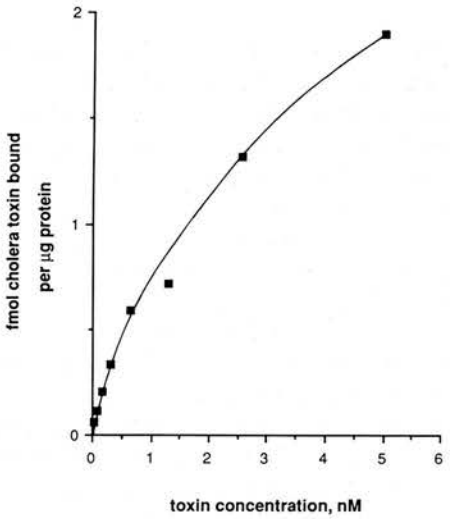


Fig.2. Binding of ^{125}I-labelled whole cholera toxin to a nuclear preparation from rabbit intestinal cells, corrected for non-specific binding.

assayed for sucrase (a marker enzyme for the plasma membrane). The activity suggested that contamination could not have been more than 3% of the protein in the preparation; quite inadequate to account for the toxin binding. Efforts to identify a specific binding protein by 'Western blot' analysis of the membrane preparation were not successful.

4. DISCUSSION

The experiments reported in this paper showed potential binding sites for cholera toxin associated with the nucleus. It seems likely that these sites are ganglioside GM1, the only receptor for the toxin that has been positively identified. This conclusion is supported by our observation that the binding is inhibited when toxin is preincubated with ganglioside and that the distribution in the cell of sites that bind anti-GM1 antibodies parallels that of toxin-binding sites. It is true that these antibodies did not interfere with the binding of toxin, but that could be easily explained if, as is probable, the affinity of the antibody for GM1 is lower than that of the toxin, or if the anti-GM1 does not bind to all the possible sites.

It is still possible, however, that the binding site is not a ganglioside, but, for example, a glycopro-

tein with a similar structure. Evidence from cell fractionation experiments, e.g. [14] has shown gangliosides in some intracellular membranes, but has usually been interpreted as showing no sign of gangliosides in the nucleus. On the other hand, binding of cholera toxin is probably the most sensitive available probe for ganglioside GM1.

Our experiments do not imply that these nuclear-binding sites can be reached by the toxin when it is working in the intact cell or in vivo, nor do they in themselves suggest any physiological role for binding to the nucleus.

Experiments on internalization of peroxidase-conjugated or ^{125}I-labelled toxin into intact cells have shown the toxin to be associated with a number of intracellular organelles, e.g. the Golgi complex [15] and other intracellular vesicles [16]. There has been no evidence for any particular binding to the nucleus. There is evidence, however, that gangliosides can be associated with cell growth and differentiation and so perhaps with gene expression in the nucleus. For example, Spiegel and Fishman [17] have shown that the interaction of the B subunit of cholera toxin with ganglioside GM1 can act as a bimodal growth regulator of cells, acting synergistically with epidermal growth factor.

Acknowledgements: M.E.P. has a CASE research studentship from the Science and Engineering Research Council of Great Britain and in cooperation with Unilever Research.

REFERENCES

[1] Lai, C.Y. (1986) Adv. Enzymol. Relat. Areas Mol. Biol. 58, 110–140.
[2] Hansson, H.A., Holmgren, J. and Svennerholm, L. (1977) Proc. Natl. Acad. Sci. USA 74, 3782–3786.
[3] Ackerman, G.A., Wolken, K.W. and Gelder, F.B. (1980) J. Histochem. Cytochem. 28, 1100–1112.
[4] Montesano, R., Roth, J., Robert, A. and Orci, L. (1982) Nature 296, 651–653.
[5] Roth, J. (1983) in: Techniques in Immunocytochemistry (Bullock, G.R. and Petrusz, P. eds) vol.2, pp.217–284.
[6] Tapia, F.J., Varndell, I.M., Prober, L., De Mey, J. and Polack, J.M. (1983) J. Histochem. Cytochem. 31, 977–981.
[7] Van Heyningen, S. (1976) J. Infect. Dis. 133, S5–S13.
[8] Bloebel, G. and Potter, V.R. (1966) Science 154, 1662–1665.
[9] Harris, J.R. and Milne, J.F. (1974) Biochem. Soc. Trans. 2, 1251–1253.
[10] Kauffmann, S.H., Gibson, W. and Shaper, J.H. (1983) J. Biol. Chem. 258, 2710–2719.
[11] Dahlqvist, A. (1968) Anal. Biochem. 22, 99–107.
[12] Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) Biochem. J. 89, 114–123.
[13] Griffiths, S.L., Finkelstein, R.A. and Critchely, D.R. (1986) Biochem. J. 238, 313–322.
[14] Matyas, G.R. and Morré, D.J. (1987) Biochim. Biophys. Acta 921, 599–619.
[15] Joseph, K.C., Stieber, A. and Gonatas, N.K. (1979) J. Cell Biol. 81, 543–554.
[16] Janicot, M. and Desbuquois, B. (1987) Eur. J. Biochem. 163, 433–442.
[17] Spiegel, S. and Fishman, P.H. (1987) Proc. Natl. Acad. Sci. USA 84, 141–145.